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Lipemia Clearing in Peptone and Anaphylactic Shock.* (21750)

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It has been shown that the optical density of an alimentary lipemia can be reduced by the intravenous injection of heparin or any of several heparin-like substances(1,2). It is assumed that a similar mechanism is responsible for the physiological clearing of a postprandial lipemia in the normal organism. However, complete proof of this is yet wanting. An attempt to demonstrate lipemia clearing factor formation by the release of endogenous heparin was made by Levy and Swank (3). Anaphylactic shock was induced in lipemic dogs and observations made of the *in vivo* changes in optical density. They found that clearing occurred in only 4 of 10 shock experiments where a rise in blood heparin was demonstrable. In some experiments they found an actual increase in the visible lipemia during shock. Later Havel and Boyle(4) performed similar experiments in dogs shocked with peptone but did not test for clearing *in vivo;* instead, they used a lipemic substrate and tested for *in vitro* clearing only. These investigators concluded that lipemia clearing factor was produced to a significant degree in every animal in shock but to a less degree than would be produced by an equivalent amount of parenteral heparin.

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TABLE I. Summary of Data on Lipemia Clearing Factor Activity, *In Vivo* and *In Vitro* in Dogs with Anaphylactoid Shock.

Shock agent	No. of exp.	Clotting time prolonged in	Significant lipemia clearing factor activity in: Dogs with prolonged clotting time		Dogs without prolonged clotting time	
			In vivo	*In vitro*	*In vivo*	*In vitro*
Peptone	7	6	2	6	0	0
Egg albumin	3	2	0	2	0	0
Totals	10	8	2	8	0	0

This paper represents an attempt to reconcile these apparently different findings and offers further evidence that endogenous heparin released during anaphylactic and peptone shock results in lipemia clearing factor formation.

Procedure. Ten mongrel dogs were used in these experiments. An attempt was made to induce shock in 7 dogs by the injection of Witte's peptone and in 3 dogs by egg albumin sensitization. Alimentary lipemia was produced by a fatty meal of 50 ml olive oil 4 to 6 hours prior to shocking. Blood samples were drawn into sodium citrate and obtained immediately before shocking and 5 minutes after shocking in all animals. Samples were obtained 10 and 30 minutes after shocking in dogs which did not die before that time. Clotting times were determined by the Lee-White method. Plasma aliquots from these samples were diluted 1:9 in normal saline, and the optical density read in a Beckman Model B Spectrophotometer at 550 mμ. After determining the *in vivo* optical density, the diluted samples were incubated at 37°C over a 6-hour period and followed for *in vitro* clearing. Shock produced by Witte's peptone was accomplished by rapidly injecting intravenously 3 ml/kg of a 10% solution in normal saline. Anaphylactic shock was produced by a rapid intravenous injection of 3 ml/kg of a 20% solution of fresh egg albumin in normal saline 30 days following a 14-day period of sensitization. (Sensitization was produced by alternate subcutaneous and intravenous injections of 0.5 ml/kg of a 20% egg albumin solution given as a single injection daily for the 14-day sensitization period.) Protamine sulfate (50 mg) was injected intravenously at the height of shock into one animal in each of the above series. A Leitz Greenough stereoscopic binocular dissecting microscope was employed to observe blood vessels of the bulbar conjunctiva in five animals during shock.

Results. A summary of *in vivo* and *in vitro* clearing is presented in Table I. Representative experiments are shown in Fig. 1 and Fig. 2. Six out of 7 dogs injected with peptone showed satisfactory evidence of shock with blood coagulation time being longer than 24 hours. One dog showed no evidence of shock and the blood coagulation time remained normal. Two out of the 3 egg albumin sensitized dogs showed satisfactory shock with greatly prolonged coagulation time, while one dog showed no evidence of shock and had a normal coagulation time. The 2 dogs which showed no evidence of shock did not clear significantly *in vivo* or *in vitro*. Only 2 of the 8 dogs which did shock showed significant *in vivo* clearing, and clearing in these 2 followed the retarded pattern seen in an hypoxic animal(5). *In vitro* clearing, however, occurred at an approximately normal rate and degree in samples from all 8 animals which evidenced shock. Almost no *in vitro* clearing occurred in the samples from the 2 animals given protamine sulfate.

Of the 5 dogs whose conjunctival blood vessels were observed 3 showed symptoms of shock and two did not. In the 3 shocked animals clumps of red cells were noted in the greatly slowed circulation, the latter appearing similar to that described in the pulmonary circulation during anaphylaxis by Burrage and Irwin(6). Of the 2 dogs in which shock was not produced, the slowing of circulation and the clumping phenomenon were very minimal and transient.

Discussion. It is assumed that the *in vitro* clearing which occurred in these experiments is the result of lipemia clearing factor formed

FIGURE I
Clearing Of An Alimentary Lipema During Anaphylactoid Shock, Representative Of The 8 Dogs Which Showed IN VITRO Clearing But No IN VIVO Clearing.

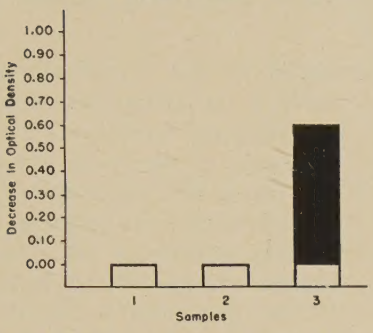

FIGURE II
Clearing Of An Alimentary Lipema During Anaphylactoid Shock, Representative Of The 2 Dogs Which Showed IN VIVO Clearing As Well As IN VITRO Clearing.

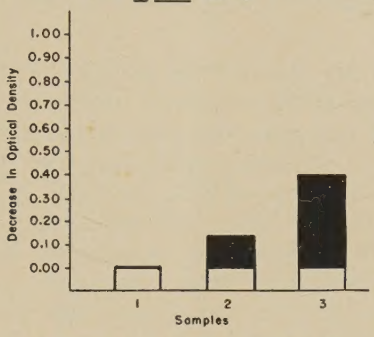

FIG. 1 and 2. Sample 1, plasma optical density prior to shock and incubated for 6 hr (for control). Sample 2, plasma optical density 10 min. after shock (for *in vivo* clearing). Sample 3, same as sample 2 after 6 hr incubation (for *in vitro* clearing).

in vivo by the heparin or heparin-like substance released during anaphylactic shock. The observation that *in vitro* clearing occurred in every animal successfully shocked is in complete agreement with the findings of Havel and Boyle(4). On the other hand, the observation that minimal clearing, no clearing or actual increased optical density occurred *in vivo* in these same animals supports the findings of Levy and Swank(3). Recent work in this laboratory suggests a possible explanation for this apparent paradox(5). It has been found that hypoxia consistently inhibits *in vivo* clearing even after intravenous heparin; however, it apparently does not inhibit *in vivo* clearing factor formation. Lipemic dogs made hypoxic and given heparin showed little clearing or actual increase in lipemia; yet, plasma aliquots from the same animals showed nearly normal *in vitro* clearing when incubated with lipemic substrate. Thus it seems possible that the hypoxia, which is usually present in anaphylactic shock(6), may have been responsible for the failure of *in vivo* clearing reported by Levy and Swank.

The possibility that peptone or egg albumin *per se* effects *in vitro* clearing is eliminated by the absence of *in vitro* clearing in either of the animals in which shock did not occur. Also, the fact that almost no clearing occurred in the samples following protamine sulfate suggests heparin or a heparin-like substance was responsible for clearing factor formation.

Conclusions. Lipemia clearing factor was formed *in vivo* during peptone and anaphylactic shock, presumably as the result of the release of heparin or a heparin-like substance. It is suggested that hypoxia inhibits the *in vivo* activity of lipemia clearing factor but does not affect its *in vitro* activity.

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Effect of Drugs on Myokinase Activity of Coronary Arteries.*† (21751)

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Previously we reported the presence of a phosphokinase in beef coronary arteries(1). A method of estimating this enzymic activity was described. Its characteristics revealed it to be similar to the myokinase previously described for rat liver, kidney and skeletal muscle(2-5). In exploring the mechanism of action of vasodilating drugs, the effect of these agents and other drugs upon this enzyme system has been studied.

Method. The beef coronary artery homogenate was made essentially as described previously. We have modified the method, however, in the interest of obtaining a higher and more uniform enzyme activity. The frozen tissue pieces from 3 to 4 beef hearts were moderately powdered in a stainless steel mortar at dry ice temperature to yield a uniformly mixed powder. An apparatus was constructed employing a high speed (30,000 R.P.M.) die grinder equipped with 2 especially designed steel razor blades. The use of this equipment gave reproducible values for enzyme activity employing the technic previously reported. The most satisfactory enzyme preparations for inhibitor studies were made by centrifuging the homogenate at 3,000 R.P.M. for 30 min. at 0°C. The slightly turbid supernatant fluid from a 5% homogenate was drawn off and used in these studies; this contained water-soluble proteins and the active enzyme. Ultra centrifuge studies with the homogenate demonstrated that further purification of the enzyme by this method was not feasible and therefore no attempt was made to prepare a mitochondrial fraction. Prior to incubation 2 volumes of the tissue enzyme preparation were mixed with one volume of a solution of the compound to be tested so that the resulting concentration of the drug was 0.02 M. The mixture was allowed to stand 15 min. at 0°C and then added to the other reactants for incubation. The final concentration of the inhibitor during incubation was 0.01 M. All determinations were made in triplicate with enzyme activity controls without inhibitor and with unincubated controls containing all the reactants and the inhibitor.

Results. Twenty-three substances were studied for their effect upon the myokinase activity of coronary artery tissue. Unfortunately some of the drugs most active as vasodilators and vasoconstrictors, such as theophylline ethylene diamine and arterenol, could not be studied because they interfered with absorbancy readings at 258 mμ in the spectrophotometer. Several potentially interfering substances were removed as insoluble perchlorates during protein precipitation and therefore did not invalidate the method. Not any of the compounds studied increased significantly the activity coefficient $\left(\frac{\text{AMP}}{\text{ADP}}\right)$. When an effect was observed it was one of inhibition, *i.e.*, inhibition of the phosphate transfer from ADP to ATP and AMP.

In Table I "A" is the activity coefficient $\left(\frac{\text{AMP}}{\text{ADP}}\right)$ and the data in the last column show the ratio between "A" control and "A" with the drug added. The compounds are listed in the order of their increasing effect. The results do not permit an analysis of the relative inhibitory effect on the myokinase and adenosine triphosphatase systems.

Posterior pituitary solution produced a significant inhibitory effect. Pitressin solution,

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TABLE I. Effect of Drugs on Myokinase Activity of Coronary Artery Tissue.

Drug		Micromoles AMP	Micromoles ADP	Micromoles ATP	A Control	A Drug	AC / AD
KCNS	C*	2.90	2.08	1.67	1.39		
	D	2.97	2.01	1.67		1.47	.95
Thiourea	C	3.12	2.01	1.53	1.55		
	D	3.19	1.95	1.60		1.64	.95
Nicotine	C	3.26	2.01	1.53	1.62		
	D	3.26	1.95	1.60		1.67	.97
$KClO_4$	C	3.12	2.01	1.53	1.55		
	D	3.12	1.95	1.46		1.60	.97
Acetylcholine Cl	C	3.19	3.02	1.74	1.06		
	D	3.19	3.02	1.74		1.06	1.00
Hexamethonium Br	C	3.04	2.01	1.53	1.51		
	D	3.04	2.01	1.53		1.51	1.00
Neostigmine Br	C	2.75	2.21	1.67	1.24		
	D	2.75	2.21	1.67		1.24	1.00
Amphetamine SO_4	C	3.04	2.01	1.67	1.51		
	D	2.97	2.01	1.74		1.48	1.02
Histamine PO_4	C	2.32	4.30	1.39	.54		
	D	2.17	4.23	1.53		.51	1.06
$NaNO_2$	C	2.75	3.42	1.74	.80		
	D	2.68	3.62	1.67		.74	1.09
Atropine SO_4	C	3.41	2.01	1.39	1.70		
	D	3.19	2.08	1.81		1.53	1.11
Morphine SO_4	C	3.12	3.22	1.74	.97		
	D	3.04	3.62	1.46		.84	1.15
Diphenhydramine Cl	C	3.40	2.01	1.53	1.69		
	D	2.90	2.01	2.01		1.44	1.17
Pitressin	C	2.54	2.38	1.67	1.07		
	D	2.38	2.68	1.60		.89	1.20
NaF	C	2.39	3.49	1.81	.68		
	D	1.67	4.83	1.32		.35	1.98
Pituitrin	C	3.33	2.01	1.60	1.66		
	D	2.25	3.09	1.39		.73	2.28
Papaverine Cl	C	3.33	2.75	1.81	1.21		
	D	2.32	4.97	.97		.47	2.59
Pitocin	C	2.39	2.55	1.74	.94		
	D	1.16	4.56	.83		.25	3.69
Na_2S	C	2.68	2.62	1.81	1.02		
	D	.94	5.44	.63		.17	5.90
Chlorpromazine Cl	C	3.19	2.95	1.74	1.08		
	D	.94	6.58	.28		.14	7.57
Triethanolamine Trinitrate PO_4	C	3.04	2.01	1.60	1.51		
	D	.87	5.23	.63		.17	9.09

* C = Control; D = Drug.

an active coronary artery constrictor, elicited little inhibition. Pitocin solution, however, showed a marked inhibitory effect.

Summary. Transphosphorylation reactions mediated by the adenylic acid system are involved in energy transfer for muscle activity. These experiments were designed to study the influence of vaso-active drugs upon two such systems, adenylate kinase (myokinase) and adenosine triphosphatase from coronary tissue. These results indicate that the constriction and dilatation of coronary arteries elic-

ited by these drugs do not involve the inhibition of these enzyme systems.

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Effect of Hormones on *In vitro* Metabolism of Bull Semen.* (21752)

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It has been demonstrated by Gassner *et al.* (1) that castration in the bull results in a complete loss of seminal fructose within a week and that testosterone therapy restores it promptly. The free amino acid concentration in semen is similarly affected although the pre-castration level is only partially restored by androgen treatment(2). While after vasectomy the fructose level remains normal the seminal amino acids disappear but return in part following administration of testosterone. Normal sperm transferred to seminal plasma of these animals is incapable of utilizing more than 50% of the fructose present. While testosterone treatment of the castrate improves its plasma quality somewhat, pregnenolone not only is ineffective in this respect but in addition is antagonistic to testosterone when the 2 are given together. It appears that although seminal plasma production in the castrate can be restored by androgen the quantitative and qualitative patterns of certain chemical constituents are sufficiently altered to affect the *in vitro* metabolic behavior of sperm(1). Aside from a short note by Baker *et al.*(3) that testosterone depressed oxygen consumption of bull sperm, little information is available relative to hormonal effect on sperm metabolism in general and that of steroids in particular. Lardy *et al.*(4) noted that thyroxine consistently stimulated glycolysis and depressed oxygen uptake of sperm without affecting motility. On the other hand, Schultze(5,6) observed an increase in oxygen uptake with low concentrations of thyroxine (0.3 to 4.0 μg) and a depression with high concentrations (15 to 80 μg), while Tshumi(7) found only motility of sperm to be stimulated by thyroxine.

In view of the foregoing considerations it seemed pertinent to determine the effects of various endocrine products on fructolysis, oxygen consumption and motility of ejaculated sperm. Of particular interest was to explore the possibility that the peculiar behavior of normal sperm transferred to plasma of castrated or vasectomized bulls restituted with steroids, may be due to the presence of such hormones or their metabolites.

Methods. In a series of 17 experiments the effects of the following substances were tested: Testosterone, 17-methyltestosterone, 17-methylnortestosterone, acetoxytestosterone acetate, androsterone, androstenedione, norandrostenedione, androstane-3,17-diol, pregnenolone, pregnanediol, estradiol, thyroxine, triiodothyronine and thyroglobulin. The compounds were solubilized in 95% ethanol, added to 0.125 M phosphate buffer and mixed with freshly ejaculated bull semen so that the final concentration in the 1:4.6 semen-buffer mixture ranged from 0.74 μg to 128 μg per ml of semen. In the earlier experiments the final concentration of alcohol in the buffer diluent was held at 0.48%. This was reduced to 0.29% in later trials in an attempt to eliminate any possible effects of alcohol on

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TABLE I. Effect of Steroids on Oxygen Uptake and Fructolysis of Bull Sperm.

Addition to semen	μg/ml semen	Z_{O_2} (mm^3 $O_2/10^8$ sperm cells/hr) No. runs	Avg deviation from control %	P.	Fructolysis (mg/10^9 sperm cells/hr) No. runs	Avg deviation from control %	P.
.29% ethanol	—	3	+ 1	NS	2	+ 3	NS
.48% "	—	4	− 7	NS	4	− 5	NS
Testosterone	2	2	−14	NS	2	−10	.05
	8	2	−15	.05	2	−17	.01
	32	4	− 8	.01	2	−19	"
	115	6	−18	<.01	4	−22	"
	128	4	− 8	"	2	−28	"
17-Methyltestosterone	2	2	−22	<.01	2	−15	"
	8	2	−31	"	2	−16	"
	32	4	−15	"	2	−15	"
	115	7	−24	"			
	128	4	−27	"			
17-Methylnortestosterone	115	2	− 8	.01			
Androstenedione	115	2	−26	<.01	2	−31	<.01
Norandrostenedione	115	2	− 7	.01			
Pregnenolone	32	4	+ 2	NS	2	−20	<.01
	128	4	− 8	.01	2	−23	"
	*	4	−12	"			
Androsterone	115	2	− 7	.01			
Estradiol	1	4	−21	<.01			
	8	4	+ 3	NS	2	−27	<.01
	32	4	− 3	"	2	−25	"
	*	8	−22	<.01			
17 α-Hydroxyprogesterone	115	2	−11	.01			
Androstane-3,17-diol	32	4	+ 1	NS	2	−23	.01
	128	2	−10	.01	2	−25	"
Pregnanediol	115	2	− 3	NS			
Acetoxytestosterone acetate	115	2	+ 1	"			

* Phosphate buffer saturated.

sperm behavior. Duplicate aliquots representing 0.5 ml of semen were used for the determination of oxygen uptake according to the direct method of Warburg. Similarly, duplicate aliquots of each sample were incubated at 37°C and fructolysis was estimated initially and hourly for 6 hours according to a modification(10) of the method of Mann(8). Motility and pH were determined at the same intervals.

Results. Effect of steroids on respiration. As shown in *Table I* the addition of 0.48% of ethanol to phosphate buffer slightly but not significantly decreased oxygen uptake by sperm. This effect was eliminated when the experiments were repeated using 0.29% ethanol as a solvent for the steroids. Testosterone reduced oxygen uptake significantly except at the 2 μg level while 17-methyltestosterone consistently and at all levels severely depressed respiration. A similar effect was seen with androstenedione and estradiol and to a lesser but still significant degree with pregnenolone, androsterone, 17-methylnortestosterone, norandrostenedione, and 17 α-hydroxyprogesterone. Androstane-3, 17-diol interfered with respiration at the high level but not at lower dosage. Acetoxytestosterone acetate and pregnanediol were inactive.

Effect of steroids on fructose utilization. Ethanol did not appear to interfere with fructolysis. However, the rate of fructose metabolism was severely restricted by testosterone, 17-methyltestosterone, androstene-

dione, pregnenolone, androstane-3, 17-diol and estradiol (*Table I*).

Effect of thyroactive agents. The response seen on respiration of sperm by thyroxine and triiodothyronine varied with dosage employed. Small quantities (*e.g.* 12 μg per ml of semen) stimulated while no effect or depression was noted with larger doses (*e.g.* 115 μg per ml of semen). Fructose metabolism of sperm was depressed by thyroxine (115 μg/ml semen) and less so by triiodothyronine. Smaller quantities were either stimulatory or ineffective.

Thyroglobulin in concentrations of 0.2 mg or 1.0 mg (0.66 μg or 3.3 μg thyroxine equivalents) had no effect on sperm respiration. With 5.0 mg and 25.0 mg (16.5 μg and 83 μg thyroxine equivalents) however, oxygen consumption was severely reduced in proportion to increase in dosage.

Discussion. It is of interest to note that the steroids tested more consistently depressed the rate of fructolysis by sperm than of respiration indicating an interference with the glycolytic phase rather than the oxidative phase. The fact that testosterone and particularly methyltestosterone were most depressive to both systems lends some credence to the assumption that there may be a direct relationship between the effect of these steroids and their androgenicity. The data presented show, however, that this does not hold for some of the steroids known to be not or only weakly androgenic. For example while pregnanediol and acetoxytestosterone acetate had no apparent effect on sperm metabolism pregnenolone, 17 α-hydroxyprogesterone and androstane-3, 17-diol were inhibitory at higher dosage levels. Assay data from this laboratory(9) on androgenicity of various steroid intermediates showed pregnanediol, acetoxytestosterone acetate and pregnenolone to be inactive and only a weak potency for 17 α-hydroxyprogesterone and androstane-3, 17-diol. Of interest is the fact that while the nor derivative of 17-methyltestosterone is nearly twice as androgenic as its mother compound its depressive effect on sperm respiration was not as great although still significant. On the other hand norandrostenedione, while only one-half as androgenic as androstenedione, also interfered much less with oxygen uptake than did androstenedione.

At present it is difficult to offer a reasonable explanation for the curious behavior of steroids in relation to sperm metabolism.

Summary. Effects of steroids and thyroactive substances on respiration and glycolysis of bovine spermatozoa were studied with the following results: 1. A consistent but insignificant reduction in oxygen uptake and fructolysis was seen when 0.48% ethanol was used as steroid solvent, while 0.29% ethanol had no effect. 2. 17-Methyltestosterone, testosterone and androstenedione caused a marked inhibition of respiration and fructolysis. A similar but less pronounced effect on oxygen uptake was obtained with 17-methylnortestosterone, androsterone, 17 α-hydroxyprogesterone, norandrostenedione, high levels of androstane-3, 17-diol, and estradiol. Acetoxytestosterone acetate, pregnanediol and low levels of androstane-3, 17-diol were ineffective. 3. Fructolysis was significantly reduced by androstenedione, pregnenolone, high levels of androstane-3, 17-diol and estradiol. 4. There is some indication of a relationship between androgenicity of a steroid and its interference with metabolic behavior of sperm. 5. Thyroxine and triiodothyronine were difficult to evaluate because of inconsistent stimulatory or depressant behavior which to some extent seemed to be influenced by the concentration employed. Thyroglobulin, however, showed a progressive depression of oxygen consumption with increase in dosage used.

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Fractional Analysis of Experimental Wound Fluid. (21753)

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In contrast to extensive studies on the blood plasma of the injured organism, only sparse information is available on the composition of the fluid present at the site of the healing wound. In earlier studies, it was observed that fluid obtained from subcutaneously implanted wire cylinders in guinea pigs was an excellent nutrient medium for tissue culture with apparent adult fibroblastic stimulating properties(1). The vit. C content seemed to follow a characteristic pattern during the healing cycle(2). S35 is concentrated at the site of the healing wound when labeled methionine is given the rat(3). Marked increases of plasma lipoprotein concentrations after single episodes of tissue injury and repeated trauma in the rabbit and dog have been reported(4,5). Following a burn of a normal extremity of a dog, there is an alteration of the plasma and lymph lipid patterns (6). A significant rise of plasma lipid concentrations during the first post-operative hours in human patients(7) is apparently reversed, becoming negative at 24 hours, reaching a minimum at 48 hours and returning to normal after 10 days(8). The relationship of wound fluid composition to the healing process has obvious practical application, not only in the local wound, but also in an understanding of the effect of the wound on the whole organism.

It was decided, therefore, to implant subcutaneously wire mesh cylinders in guinea pigs, harvest the accumulated wound fluid, and compare its colloidal and crystalloidal content with the blood plasma of the animal and the blood plasma of uninjured pair-fed control guinea pigs.

Methods: a) Thirty healthy male guinea pigs were divided into 2 equal groups designated A and B. Two stainless steel wire mesh cylinders were prepared and implanted obliquely(1) in the dorsal subcutaneous surfaces of each of the animals in Group A, Group B served as controls. The groups were placed in adjacent cages in an air-conditioned animal room, and the pair-feeding of Group B against Group A accomplished over a 7-day period. The pair-fed diet consisted of Purina rabbit chow and lettuce, with water allowed *ad libitum*. On the 7th day approximately 1 cc of fluid was aspirated from each of the wire mesh implants of Group A animals and pooled to constitute *wound fluid sample No.* 1. Three ml of blood were taken also from each of the Group A animals by cardiac puncture with a lightly heparinized syringe and pooled. After separation by low-speed centrifugation, the pooled plasma was designated *wound plasma sample No.* 1. Finally, 3 ml of blood was taken by heart puncture from each of the control Group B guinea pigs and pooled. After separation by low-speed centrifugation, this pooled plasma was termed *control plasma*

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sample No. 1. Each of the pooled samples was analyzed for *lipoprotein* concentration (9), the levels of *cholesterol*(10), total *protein*(11), *phospholipid*(12), and *uric acid* (13), and subjected to electrophoretic protein partition(14). These protocols were repeated over successive 8-day periods with new groups of animals until the results from 13 pooled samples of wound fluid, wound plasma, and control plasma, respectively, had been accumulated.

The means and appropriate levels of significant difference were calculated for each plasma variable and the results recorded in Table I.

b) A routine identical to that recorded in a) above, but utilizing groups of 5 wounded guinea pigs, was followed in order to determine concentrations of sodium(15), potassium(15), and chloride(16). Similarly, groups of 3 animals were used for total calcium(17), and phosphorus(18) assay, and single animals for determination of the plasma and wound fluid osmolarity(19).

The results of inorganic ion and osmotic pressure determinations are recorded as means and levels of significant difference in Table II.

Discussion. A comparison of control plasma and wound plasma data recorded in Table I indicates a lipoprotein response to trauma in guinea pigs similar to that previously observed in rabbits, dogs, and humans. The concentrations of the S_f 12-400 class of lipoproteins, of phospholipid, and of uric acid are all significantly increased in the plasma of wounded animals. Somewhat at variance with findings in the other species is the observation that plasma cholesterol concentrations remain essentially unaltered in the wounded guinea pig.

The qualitative similarity between wound fluid and plasma of wounded animals is not surprising. The single exception, the complete absence of fibrinogen, is probably related to its conversion to fibrin in the inflammatory fibroblastic process within and without the wire mesh cylinder. The marked quantitative differences between plasma and wound fluid, however, seem to be associated with changes in the character of some of the colloidal com-

TABLE I. Colloidal Fractions and Concentrations in Experimental Wound Fluid, and Plasma of the Wounded Animals and Their Controls.

Group	No. samples	No. animals /sample	S_f 0-12, mg %	S_f 12-400, mg %	Alb, %	α_1, %	α_2, %	β, %	ϕ, %	γ, %	T.P., g %	Chol., mg %	Phos.L., mg %	U.A., mg %
Control—Plasma I	13	15	58.4	34.4	49.5	8.8	19.3	5.8	8.5	7.9	6.05	49.5	2.0	1.44
Wound—Plasma II	"	"	63.0	117.8	43.4	13.1	20.6	6.1	8.5	7.8	5.88	57.1	3.4	2.88
Wound—Fluid III	"	"	11.7	4.1	44.5	11.8	23.2	10.1	.0	10.2	3.82	26.7	.6	1.66
P (I *vs* II)			NSD*	<.01	<.01	<.01	NSD	NSD	NSD	NSD	NSD	NSD	<.01	<.01
P (II *vs* III)			<.01	"	NSD	NSD	"	<.01	<.01	<.01	<.01	<.01	"	NSD
P (I *vs* III)			"	"	<.01	<.05	<.01	"	"	"	"	"	"	"

* No significant deviation.

TABLE II. Crystalloidal Concentrations in Experimental Wound Fluid and Plasma of the Wounded Animals and Their Controls.

Group	No. of samples	No. animals /sample	Na, Meq/L	K, Meq/L	Cl, Meq/L	No. of samples	No. animals /sample	Ca, Meq/L	P, Meq/L	No. of samples	No. animals /sample	O.P., milli-osm/L
Control—Plasma I	24	5	142.5	6.94	108.4	15	3	5.95	4.9	15	1	289.8
Wound—Plasma II	"	"	145.4	7.13	109.9	"	"	5.45	4.9	"	"	291.4
Wound—Fluid III	"	"	127.8	5.89	109.0	"	"	2.75	2.6	"	"	274.4
P (I *vs* II)			NSD	NSD	NSD			NSD	NSD			NSD
P (II *vs* III)			<.01	<.01	"			<.01	<.01			<.01
P (I *vs* III)			"	"	"			"	"			"

ponents. The reduction in the lipids and lipoproteins of wound fluid is recorded in spite of the fact that electrophoretic partition reveals a relatively increased proportion of the α_2 and β protein complexes. These data indicate that protein molecules that were conjugated with cholesterol or phospholipid in the plasma appear, in large proportion, devoid of their lipid components in the wound fluid. Such a conclusion seems consistent with electrophoretic measurements recorded in Table I because, although the α_2 and β proteins are to a great extent conjugated with one or more of the physiologic lipids, the uncharged lipid component makes only a negligible contribution to electrophoretic mobility.

Any conclusion relating the greatly increased proportion of the lipid-free protein moieties to the healing process would be hazardous indeed. However, it might be safe to hypothesize that high lipid concentrations and the healing process may be mutually inimical. Such would be consistent with the observations that lipid infiltration of tissue is characteristic of many degenerative diseases and that the healing process, in a sense, is the antithesis of physiologic degeneration. The relative increase in gamma globulin at the wound site is of interest, particularly with regard to the bacterial antagonism that occurs in every wound.

In the main, the data recorded in Table II have descriptive interest only. The fact that chloride, uniquely among the inorganic ions determined, remains unchanged between wound plasma and wound fluid, may have some significance. These results are somewhat at variance with those reported by Andreesen and Tammann (20) who describe an increase in potassium and calcium and a decrease in sodium and chloride with hyperosmolarity of wound fluid. The relationship of the inorganic components of the wound fluid to the healing process is receiving further study in our laboratory.

Summary. Wound fluid, contained in subcutaneously implanted wire mesh cylinders, was harvested. Its crystalloidal and colloidal components were compared with those of the blood plasma of the wounded animals and their pair-fed controls. Significant differences

were observed in the protein and lipoprotein fractions.

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Hematologic Changes in Hypothermic Dogs. (21754)

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Hypothermia as an adjunct of anesthesia has shown increasing usefulness during the past 15 years(8,10), and several studies of physiological effects of cooling have been reported(1,6,7,9,12). There has been no systematic study of the hematologic effect. A rise in hematocrit has been noted(1,7,12). Leukocytosis has also been reported(10). Clotting time of blood from hypothermic dogs carried out at 37°C has been reported to be normal(8) and prolonged(6).

Methods. The hypothermia experiments were carried out in a series of dogs using a method of surface cooling previously reported from this laboratory(9). Dogs were anesthetized with intravenous pentothal. An endotracheal catheter was inserted and connected to an automatic respirator. A polyethylene catheter was inserted into the aorta via the femoral artery. Continuous blood pressure recordings were made using a Statham strain gauge and Sanborn recorder. Thermocouples in the rectum and esophagus permitted continuous temperature recordings by a Brown potentiometer. A continuous 3-lead E.K.G. was also recorded. The animals were placed in an ice bath at 4°C and cooled until either ventricular fibrillation occurred or the body temperature reached 17°C. When fibrillation occurred 30 minutes was allowed to elapse; then the chest was opened, the heart defibrillated electrically and the animal rewarmed. If fibrillation did not occur before the temperature reached 17°C, the animal was rewarmed in the water bath without surgery. The only infusions were small doses of Anec-

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TABLE I. Changes in Blood Cell Counts in 9 Dogs during Hypothermia (Arterial Blood).

W.B.C. (1000/mm³)		Hematocrit (% packed R.B.C.)		Hgb (g/100 ml)		R.B.C. (million/mm³)		Platelet (1000/mm³)	
A	B	A	B	A	B	A	B	A	B
8.1	.75	37	53.5	—	—	5.5	8.0	105	6
13	2	35	52	12.0	18.3	5.0	6.9	170	9
8.1	1.2	42	41	14.3	14.0	4.4	4.3	251	23
11.0	1.8	44	54	14.9	18.0	5.4	7.2	241	17
11.3	2.5	41	56.5	14.3	20.1	4.6	7.5	226	7
6.1	2.7	46	55	15.9	18.7	5.8	8.1	204	65
—	—	—	—	—	—	—	—	702	44
—	—	—	—	—	—	—	—	290	33
—	—	—	—	—	—	—	—	153	30

A = Preliminary count. B = Count at time of lowest body temperature.

tine (succinylcholine chloride) given when the animals' temperatures reached about 27°C to control shivering, and occasional small infusions of saline solution to keep the arterial catheter open. Through this catheter periodic blood samples were drawn for counting and for clotting studies. Siliconized syringes were used for the latter. *Red cell counts* were carried out by the usual Thoma pipette method with Dacie's solution as diluent. Approximately 500 cells were counted. White cell counts were also carried out by standard technic. Hematocrit was determined in Wintrobe tubes. Hemoglobin determination was carried out by the cyanmethemoglobin method (5). *Platelet counts* were done by the Brecher and Cronkite(3) technic and the reticulocyte counts by the method of Brecher and Schneiderman(4) using the New Methylene Blue technic described by Brecher(2). *Glass and Silicone* clotting times were carried out by the Lee-White method, with samples placed in a 37°C water bath immediately after drawing. The one-stage prothrombin time was carried out by Quick's method and the prothrombin consumption test was carried out by the Stefanini and Crosby(11) modification of Quick's method. Bleeding time was determined on the tongue, since skin was unsatisfactory because of vasoconstriction produced by the ice water. The incision was

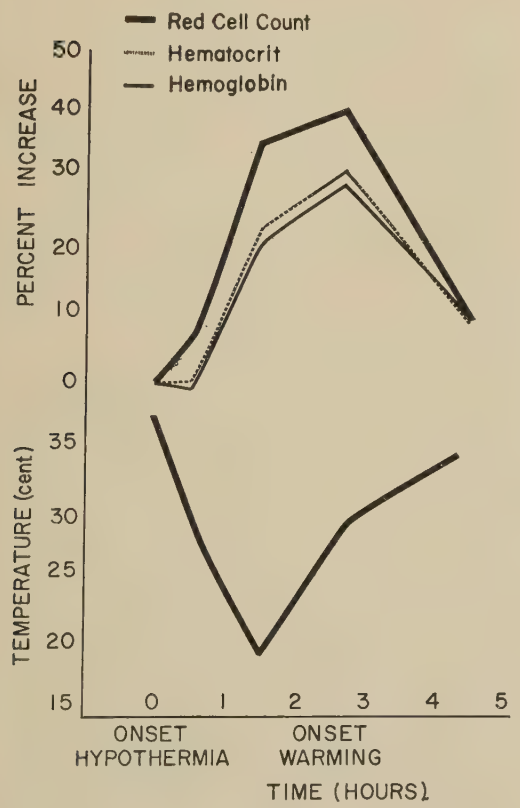

FIG. 1. Dog No. 4. Correlating changes in red count, hematocrit and hemoglobin with body temperature. This figure shows rise in red cell values with cooling and return to normal with rewarming.

TABLE II. Hemoconcentration in 6 Dogs during Hypothermia (Arterial Blood).

	% max increase R.B.C.*	% max increase Hgb.*	% max increase Hct.*
	45	—	43
	39	61	60
	66	32	28.5
	41	30	32
	82	45	41.5
	40	19	19.5
Avg	52	37.5	37.4

* While maximum changes usually occurred at height of hypothermia, they occasionally occurred in the early rewarming phase, as in dog 3. (Compare Table I with Table II.)

TABLE III. Changes in Bleeding and Clotting in 8 Dogs during Hypothermia (Arterial Blood).

Bleeding time (min.)		Glass clotting time (min.)		Silicone clotting time (min.)		Prothrombin consumption time (sec)		Clot retraction	
A	B	A	B	A	B	A	B	A	B
1½	20⅙	10	15¼	35	71	21.6	13.1	3+	0
2¼	7½	7¾	10½	23	66	22.6	9.0	4+	0
3	0*	6½	8½	22	18	17.5	10.2	4+	2+
1½	5⅔	8¾	16	29	110	24.5	11.7	4+	0
1½	3 5/12	12½	13½	32	41	20.1	10.3	4+	0
1⅓	2⅚	4	14½	7½	50	13.7	8.9	4+	2+
1	3½	6	15	16	50	70.0	9.0	4+	Trace
0	0	7	14	13	150	29.0	13.0	4+	0
—	—	—	—	—	—	—	—	—	—

A = Preliminary result. B = Result obtained at time of lowest body temperature.
* The dog was fibrillating.

made with a #11 Bard Parker blade anchored in a cork stopper with the point of the blade extending exactly 5 mm beyond the stopper. Normal bleeding time in dogs for this method is 1 to 3 minutes.

Results. Tables I and III summarize the results in each dog comparing the control levels with the results obtained at time of lowest body temperature.

1. *Red cells,* hemoglobin and hematocrit: Fig. 1 shows the results in a typical experiment (dog 4) of hemoglobin, hematocrit and red blood cells correlated with temperature changes. In this case the values continued to rise after temperature was on the way up. In other dogs the maximum rise was at or just before the maximum fall in the temperature. Table II shows the maximum increase in hemoglobin, hematocrit and red count in 6 dogs. The red cell rise averaged 52% of the preliminary value, the hemoglobin 37.5% and the hematocrit 37.4%. In general the M.C.H.C. remained constant. The M.C.V. and M.C.H. tended to fall, but values were inconstant and may have been due to error in red cell counts.

2. *White cells*: Table I shows the change in leukocyte counts comparing the preliminary values with the results obtained at time of lowest body temperature. Every dog showed a striking drop, and the average decrease for all dogs was 80.1%. In Fig. 2 the response in a typical experiment (dog 4) is shown.

3. *Blood smears* were made in 6 dogs. One dog only showed a relative increase in lymphocytes. The other dogs showed no significant trend. One had an increase in eosinophiles from 4% before cooling to 21% at the height of hypothermia. All dogs showed many nucleated red cells beginning at the time of maximum hypothermia and becoming more striking during early warming. The least response was 8 nucleated red cells per 100 leukocytes, the most marked was 21 and the average for the six dogs during the early warming period was 12.

4. *Reticulocyte counts* were carried out in 3 dogs. The maximum response was seen during the early warming period, and was 1.03%, 0.7%, 1.5% respectively in dogs number 4, 5, and 6.

5. *Platelet counts* and clotting studies: In each of the 9 dogs the previously normal platelet counts dropped to levels between 6,000 and 65,000 per cu mm at the time of maximum cooling. With the drop in platelets the bleeding time and the silicone clotting time became prolonged, prothrombin consumption became poor and clot retraction decreased or disappeared (Table III). Fig. 2 illustrates the results in dog 4. It is of interest to note the increased coagulability which followed the return of platelets toward normal. The bleeding time and silicone clotting time were both much shorter than the control levels.

In the 3 dogs in which it was followed plasma prothrombin activity showed no change. Fibrinogen and fibrinolysin studies

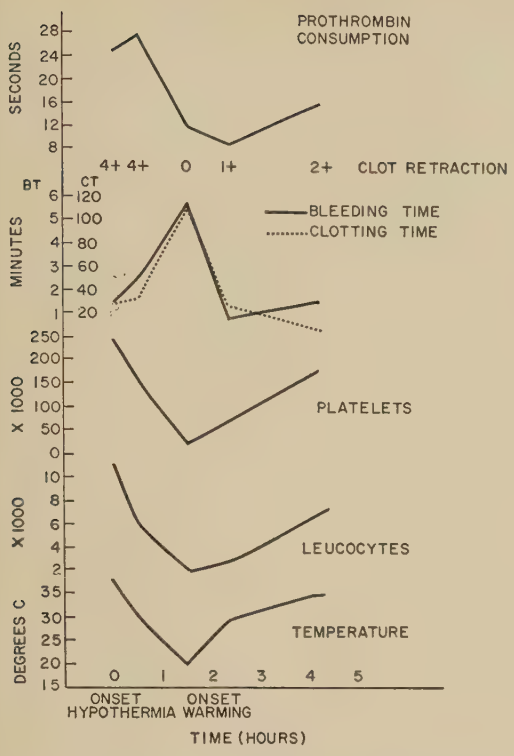

FIG. 2. Dog No. 4. Correlates changes in white cell, platelets, silicone clotting time, bleeding time, clot retraction and prothrombin consumption with body temperature. As the platelets drop all various platelet functions are interfered with. Figure also shows hyper-coagulability in 2¾ hr test of bleeding time and in 4½ hr test of silicone clotting time.

were carried out in one animal (dog 8). There was a slight decrease in fibrinogen level during cooling (preliminary value 620 mg per 100 ml, at maximum cooling 449 mg). Qualitative and quantitative fibrinolysin determinations revealed no evidence of fibrinolytic activity. The bleeding tendency in this dog was no different from the others.

Discussion. The hematologic response to cold was consistent in the dogs tested. The pattern of response was as follows: 1. The red count, hemoglobin and hematocrit all rose. The M.C.H.C. remained fairly constant. The M.C.V. and M.C.H. showed inconsistent changes due possibly to the wide range of error in the red blood cell count. The cause for the increase was not clear but the most probable explanation is hemoconcentration due to a temporary withdrawal of a portion of the plasma from active circulation. This concept has been supported by a single experiment in which plasma and red cell volumes were measured.

2. Leukocytes disappeared almost completely. The decrease involved all the cell types equally, with one exception as noted. Within a half hour of rewarming the leukopenia disappeared. It is not clear what caused the decrease or subsequent increase in white cells. A basic question is whether the cells that appeared after rewarming were the same ones that disappeared. The absence of shift to the left of leukocytes in the warming phase suggests that these are not new cells.

3. Platelet and clotting studies. The dogs exposed to hypothermia showed a nearly complete disappearance of platelets. This thrombocytopenia seems adequate to explain the associated prolongation of silicone clotting time, decrease in prothrombin consumption, loss of clot retraction and increase in bleeding time. The bleeding would probably be worse were it not for the extreme sluggishness or even standstill (when the dogs fibrillate) of the circulation. Studies now in progress indicate that the rise in platelet count in the warming phase represents a reappearance of old platelets and not a production of new ones.

During the recovery phase there was a decrease in silicone clotting time and bleeding time, in many cases to values much shorter than the controls. This hyper-coagulability cannot be explained by any change in platelet number or plasma prothrombin activity. The hypothermic dogs showed no significant change in plasma prothrombin activity, fibrinogen levels, or fibrinolytic activity.

It should be borne in mind that the results reported here were obtained from animal experiments. Whether the same changes occur in hypothermic humans we do not yet know. There may be species differences and, furthermore, humans are not usually cooled to the very low temperatures obtained in these dogs.

Summary. In 9 hypothermic dogs cooled below 20°C the following hematologic changes occurred: 1. Red cell count, hemoglobin concentration and hematocrit rose. 2. White cells of all types nearly disappeared. 3.

Platelets nearly disappeared. With the thrombocytopenia the dogs developed prolonged silicone clotting times, loss of clot retraction, poor prothrombin consumption and prolonged bleeding time. 4. During the early warming period the platelets and white cells returned to normal. The clotting studies also returned to normal except the silicone clotting time and the bleeding time which showed evidence of hyper-coagulability. 5. The red count returned to normal somewhat more slowly. During the early warming period nucleated red cells appeared in the peripheral blood.

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Sickling, A Quantitatively Delayed Genetic Character.* (21755)

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Although sickle cell anemia is a not uncommon hereditary disorder of Negroes, its occurrence in early infancy is extremely rare, and, as a cause of death *in utero,* it is unknown. Attempting to explain the scarcity of this disease in early life, Watson *et al.*(1), compared "moist coverslip" sickling preparations from the blood of 226 newborn Negro babies with similar preparations from the mothers. The incidence of sicklemia in both groups was the same, but the percentage of sickling erythrocytes in the babies was considerably smaller than in the mothers. In a detailed study of one infant, the percentage of sickling gradually increased with age, until at 4½ months, there was 90%, in comparison with 6% at birth. Because 4½ months is the age that marks the disappearance of practically all of the fetal type of hemoglobin, Watson suggested that the differences in the sickling phenomenon of infants and adults are due to a chemical difference between fetal and adult types of hemoglobin. Similar data have been presented by Scott *et al.*(2) and Allison(3). Shortly after the publication of Watson's report, Pauling *et al.*(4) found that sickling erythrocytes contain an electrophoretically distinct type of hemoglobin, and Perutz and Mitchison(5) reported that this sickling (S) hemoglobin in the reduced form has an extremely low solubility. Under conditions of diminished oxygen tension, there is an orderly precipitation of reduced S hemoglobin molecules(6) in the form of tactoids, and this appears to be the physical-chemical basis of the sickling phenomenon. All those erythrocytes containing S hemoglobin in a critical concentration of 20%(7), and only those, are capable of sickling. *Fetal* (F) *hemoglobin,* at birth the predominant type, is in normal subjects gradually replaced by adult (A)

* This investigation was supported by a U. S. Public Health Service grant.

TABLE I. The Percentage of Sickling Erythrocytes and of F and S Hemoglobin in Infants with Sicklemia.

Name	Age	% sickling erythrocytes	% S Hgb (from scanning diagrams)	Approximate* % S Hgb (paper electrophoresis)	% F Hgb (alkali denaturation)
CAL	N	13	14	Not demon.	63
	2 wk	25	24	Small amt	58
	6 "	64	27	*Idem*	44
	7 mo	97	51	50	2
PN	N	Small†	12	Not demon.	68
	4½ mo	Large†	Not done	50	4
AC	N	9	*Idem*	Not demon.	63
	7½ mo	97	"	50	3
SD	N	5	"	Not demon.	76
	7 mo	97	"	Not done	Not done
CM	N pre‡	3	Not demon.§	Not demon.	81
	4½ mo	97	Not done	40	22
BB	N	3	*Idem*	Not demon.	75
CEW	N	15	21	Small amt	63
	3½ wk	32	Not done	*Idem*	49

* Approximate percentages determined by visual comparison. Percentile estimations of S hemoglobin were not attempted when the predominant type of hemoglobin was F.
† No exact count made.
‡ Premature.
§ Not demonstrable.
N = Newborn cord blood.

hemoglobin, and full production of the latter is not achieved until the age of about 4½ months. A similar delay in the full production of S hemoglobin, if such could be demonstrated in subjects with sicklemia, might well explain the scarcity of sickling erythrocytes in early life.

To test this hypothesis, cord blood from Negro babies was examined for sickling and the hemoglobin analyzed for S and F types. Whenever possible, percentages of sickling erythrocytes and of S and F hemoglobin were determined in successive blood samples from the babies with sicklemia.

Methods. Sickling preparations were made with metabisulfite(8). The coverslips were sealed with liquid paraffin, allowed to stand for several hours, and the percentage of sickling determined by counting 1,000-2,000 cells under 400x magnification. Later preparations were made with the gas chamber method of Hahn and Gillespie(9). Good agreement was found between the two methods. Free and paper electrophoresis analyses were performed by methods previously described(10). Slight modifications of the latter will be described in detail elsewhere. F hemoglobin was determined by the method of alkali denaturation of Singer and Chernoff(11).

Results. Blood samples (all but 3 were cord blood) were obtained from 84 newborn Negro babies. Positive sickling was found in 7 (8.3%) of these, with 3-15% sickling cells. No appreciable quantity of S hemoglobin could be detected by paper electrophoresis in any of the hemoglobin samples of the 7 newborn babies with sicklemia. In free electrophoresis of 4 of these, the S hemoglobin content ranged from "not demonstrable" to 21%. In contrast, blood samples from 4 of the same babies at 4½ to 7 months showed almost 100% sickling, and the S hemoglobin values were of the order of 40-50%, as in adults with SCT(12). These data are summarized in Table I and some of the paper electrophoresis patterns are reproduced in Fig. 1.

Blood samples from one baby (C.A.L.) with sicklemia were studied at birth, 2 weeks, 6 weeks and 7 months respectively. The scanning diagrams of the hemoglobin samples from this baby are reproduced in Fig. 2, and the data on the increasing percentage of sick-

Age	Diagnosis	Hemoglobin types	% F Hgb by alkali denaturation
Adult	Normal (control)	A	1
N	*Idem*	FA	80
N	SCT	SFA	63
Idem, 3 wk	"	"	49
7 mo	"	SA	2
7 "	"	"	3
6 yr	SCA (control)	SF	7

Hemoglobin samples, .006 ml of 12-14%. Veronal buffer, pH 8.6, μ .05.
SCT—Sickle cell trait. SCA—Sickle cell anemia.
N—Newborn cord blood.

FIG. 1. Paper electrophoresis patterns of hemoglobin of infants with sickle cell trait.

ling erythrocytes in relation to the increase of S and the disappearance of F types of hemoglobin are presented in Table I. Data on one premature baby (C.M.) with sicklemia are also included in Table I. This baby had 3% sickling erythrocytes at birth and S hemoglobin was not demonstrable in free nor paper electrophoresis. At 4½ months virtually all of the erythrocytes sickled, and S hemoglobin by paper electrophoresis was of the order of 40% even though about 20% of the total hemoglobin was still of the F type. Further study may provide information about the distribution of S, F and A types of hemoglobin in the erythrocyte population in infancy. At any rate, the paucity of sickling erythrocytes and the rarity of SCA before the age of about 4½ months are adequately explained by the fact that full production of S hemoglobin does not occur before this age.

Several other abnormal types of hemoglobin have been described, of which hemoglobin C is the most common (13). One cord blood sample in the present series contained a small amount of hemoglobin C by paper electrophoresis. In free electrophoresis (Fig. 3), the hemoglobin C content of this sample was about 8%. This figure contrasts with the values of 30-40% hemoglobin C found in adult subjects with hemoglobin C trait (13) and suggests that hemoglobin C, like hemoglobin S, is an adult type, not fully formed at birth.

Summary. Positive sickling was found in 7 or 8.3% of blood samples from 84 newborn Negro babies, with 3 to 15% of erythrocytes capable of sickling. In free electrophoresis of

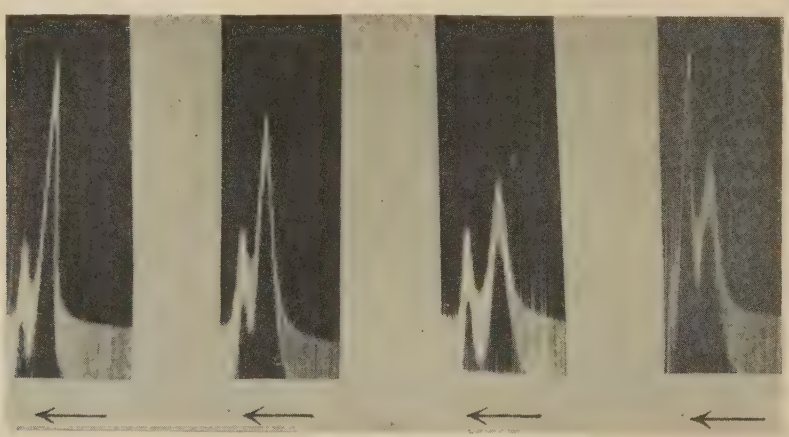

FIG. 2. Scanning diagrams of hemoglobin of C.A.L. at birth, 2 wk, 6 wk and 7 mo. Ascending patterns of about 1% carboxyhemoglobin solutions in cacodylate buffer pH 6.5, μ 0.1.

FIG. 3. Scanning diagram of hemoglobin of newborn baby with hemoglobin C trait. Ascending pattern of 1% carboxyhemoglobin solution in cacodylate buffer pH 6.5, μ 0.1.

the hemoglobin of 4 of these 7, the S hemoglobin content ranged from "not demonstrable" to 21%. Blood samples from one baby with sicklemia were studied at birth, 2 weeks, 6 weeks and 7 months respectively, with a progressive increase of sickling erythrocytes of from 13% at birth to 97% at 7 months, and a corresponding increase in S hemoglobin of from about 14 to 50%. Three of the other babies with sicklemia were reexamined at 4½ to 7 months, at which time almost 100% of the erythrocytes sickled and S hemoglobin values were of the adult order of 40 to 50%. The presence of hemoglobin C trait was detected in the blood of one newborn baby of the 84, with a hemoglobin C content of only 8%. The rarity of sickle cell anemia and the paucity of sickling erythrocytes in young infants are explained by a quantitative delay in the production of S hemoglobin which, like normal adult hemoglobin (and probably hemoglobin C), does not reach adult levels until the age of about 4½ months.

We are indebted to John A. Cudd and Bruce D. Genereaux for the free electrophoresis analyses and to Mary Lou Fraser for technical assistance.

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Experimental Induction of Periarteritis Nodosa in White Rats. (21756)

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Numerous investigators have demonstrated that estrogens exert a deleterious effect on the reproductive systems of male vertebrates (Moore and Price(1), Zondek(2), and Selye and Friedman(3)). Perry(4) has shown that estradiol diproprionate, when administered in doses of 0.1 mg to male rats at 3 day intervals for 30 days, is markedly deleterious. Body weight diminishes greatly, spermatogenesis is inhibited, and testosterone secretion is checked.

This investigation was undertaken to determine whether follicle stimulating hormone (FSH) would restore damaged gonadal activity in estrogen injured male rats. FSH was found to restore lost weight, but in place of normal gametogenic tissue recovery it stimulated proliferation of large atypical cells in testis tubules. These cells gradually disappeared during the post treatment period, and an ultimate fibrosis of seminiferous tubules developed (Fig. 1,2). Within 4 months after treatment interstitial arterioles of the testis exhibited characteristic changes of periarteritis nodosa (Fig. 1, 2). From 6 to 12 months after treatment distinctive signs of periarteritis, both macroscopic and microscopic, appeared in small arteries in many areas of the body. *Periarteritis* has been considered as due to infections, toxins, viruses, and allergies. More precise causes have been found in laboratory animals. Rich and Gregory(5) reported development of periarteritis in rabbits after treatment with sulfanilamide. Selye(6) and his associates showed that it rapidly develops in rats sensitized by unilateral nephrectomy, given high salt and protein diets, and treated with certain pituitary and adrenal hormones. This investigation indicates that periarteritis may also result from hormonal imbalances involving gonadal, pituitary, and adrenal cortical hormones. However, it does not appear until several months after treatment.

Procedure. Male rats of the Sprague-Dawley strain between 10 and 12 months of age were given a standard diet of Arcady pellets, occasional feedings of lettuce, and water *ad libitum*. All animals were periodically weighed throughout the observation period. Thirty-eight animals were used in this investigation. Six controls were given estrogen alone and 6 were given FSH alone. Five animals served as untreated controls. Twenty-one animals were given both estrogen and FSH, 3 of which died during the year following treatment possibly due to periarteritis. Subcutaneous injections of 0.1 ml estradiol diproprionate (Ovocylin—Ciba—1 mg per ml) were given every third day until 10 injections had been given. Thereafter similar injections of 0.1 ml FSH (Armour—300 γ per ml) were given every third day until 10 had been given. In a personal communication Steelman(7) stated that slight amounts of luteinizing and thyrotropic hormones were the only contaminants of FSH. After the 60 day injection period, no further treatment was employed; but all animals were closely observed during ensuing months. Individual experimental and control animals were sacrificed at 0, 1, 2, 4 to 6, and 8 to 12 months post treatment. Gross and microscopic findings are discussed below.

Observations. Controls. At autopsy untreated controls failed to exhibit testicular pathology. There was no evidence of dietary deficiencies. Estrogen treated controls did not exhibit periarteritis either in testicular or other arteries. Spermatogenesis was absent in these controls in early months but had resumed in the 8 to 12 month post treatment animals. Controls that received only FSH failed to exhibit periarteritis in testicular or other vessels. Spermatogenesis persisted during the entire period of observation.

Experimental animals autopsied prior to 4 months post treatment. It was observed that FSH stimulated atypical mitotic divisions in gametogenic tissue of animals whose germinal epithelia had been damaged by previous estrogenic treatment. Hypertrophy of interstitial tissue was present in these animals,

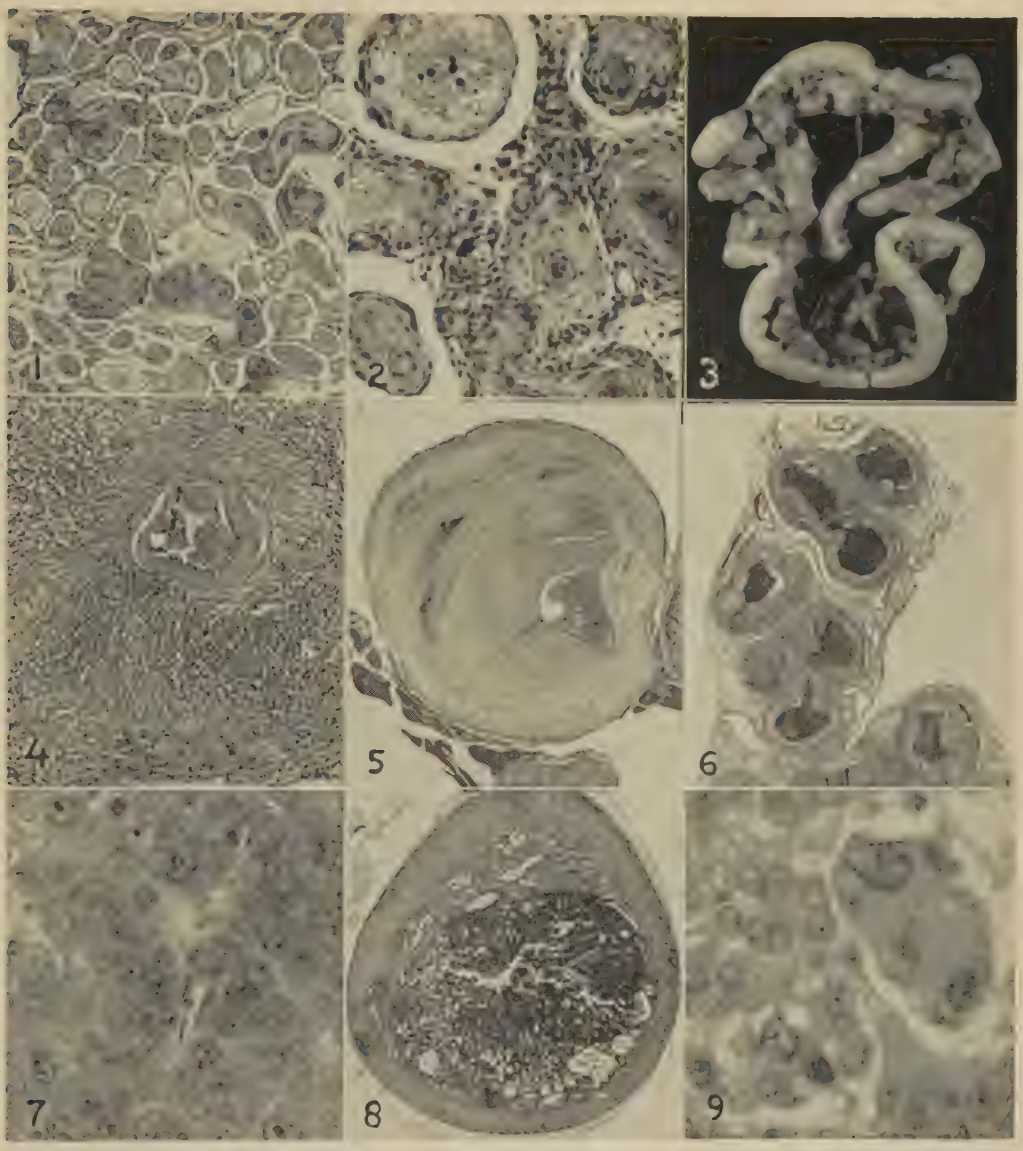

FIG. 1. Extensive area of testis showing atrophic seminiferous tubules and periarteritis in interstitial arterioles. 34 ×.

FIG. 2. Testicular vessels of animal referred to in Fig. 1. Note sparsity of nuclei in media, collagenous fibers in walls, and atrophic tubules. 293 ×.

FIG. 3. Portion of intestinal region of rat with extensive periarteritis. Note nodules and expanded arteries. About one-half normal size.

FIG. 4. Section of single large nodule from animal autopsied at 8 mo interval showing necrosis of intimal and subintimal layers, partial absence of elastica interna, and pronounced inflammatory reaction with necrosis in additional layers. 67 ×.

FIG. 5. An advanced stage in a mesenteric nodule showing thrombosis and sclerosis. 22 ×.

FIG. 6. Section through mesenteric arteries of animal with extensive periarteritis. 22 ×.

FIG. 7. Large tumor cells in adrenal gland from an animal in the 8 mo group. 293 ×.

FIG. 8. Section of adrenal gland from animal of the 12 mo group. Note cortical nodules large dark hyperplastic mass, and sinusoids filled with fibrin and amorphous material. Medullary tissue is compressed between the cortex and hyperplastic mass. 22 ×.

FIG. 9. One giant and many tumorous eosinophilic cells from the pituitary of an animal in the 6 mo group. 293 ×.

but no signs of periarteritis were present. Lymph nodes in many areas of the body were hypertrophied.

Experimental animals autopsied 4 to 6 months after treatment. In these animals the above mentioned atypical gametogenic cells undergo deterioration, and the first evidences of periarteritis appear. Many small blood vessels, mostly arterioles, of the interstitial area have become greatly enlarged. In sections they commonly exhibit a small lumen filled with endothelial cells and an eosinophilic amorphous material; and they have thickened disorganized walls. These walls show fibrinoid necrosis beginning in the intima and subintima. An inflammatory reaction is present throughout, particularly in the adventitia. In initial stages polymorphs are numerous; but soon the walls become granulomatous and contain many fibroblasts, plasma cells, and occasional eosinophiles. Many vessels also come to exhibit thrombosis, fibrosis, and sclerosis. Such vessels are acidophilic, and the nuclear population is sparsely scattered in a collagenous matrix (Fig. 1, 2). In these animals there were no indications of periarteritis outside the testes, but lymphoidal hyperplasia was conspicuous in many areas.

Experimental animals autopsied 8 to 12 months after treatment. Animals in this group moved with difficulty, had bloated abdomens, and appeared generally cachectic in marked contrast to all control and other experimental animals. The testes of these animals were of normal size but were hard on palpation. Seminiferous tubules were atrophic. Their basement membranes were fibrotic, germinal cells were absent, and few atrophied Sertoli cells remained. Periarteritis was conspicuous and extensive in interstitial arterioles. The most striking observation in these animals was the presence of numerous large nodules in the ordinarily small mesenteric arteries all along the intestinal border from the stomach to the rectum. Small arteries in the mesentery itself were greatly enlarged and nodular (Fig. 3). Their walls were characterized by cellular proliferations and their lumina were filled with eosinophilic amorphous material (Fig. 4). Histological reactions in nodules and vessels range through all stages described earlier in testicular arterioles. In these instances, however, thrombosis, fibrosis, and sclerosis were more pronounced (Fig. 5, 6).

Small arteries in the pancreatic, splenic, and thymic areas exhibited the same changes found in mesenteric vessels. Numerous thin walled vessels within the kidneys were filled with an eosinophilic hyaline-like material. Pulmonary arterioles, however, were unaffected. Lymphoidal hyperplasia remained extensive. The thymus, while usually atrophic, was sometimes hyperplastic. These findings are consistent with certain observations of Selye(8). Megakaryocytes and nucleated red cells were evident in splenic sinusoids.

The *adrenal glands* of animals in all groups in which periarteritis was present were hypertrophied ranging from 5 to 10 mm in diameter as compared to 3 mm for controls. The glands were hyperplastic and adenomatous. Numerous cells with large nuclei and prominent nucleoli have been considered tumorous (Fig. 7). The hyperplasia appears to be cortical. Some sinusoids of the gland were greatly distended with a reddish granular precipitate. Fig. 8 is typical for adrenal hyperplasia. Anterior pituitary glands exhibited hypertrophy up to 5 times normal size. Extensive strands of connective tissue separated glandular areas and fibrinlike material was present in enlarged sinusoids. Many large degranulated eosinophiles having a hyalinelike cytoplasm and similar to those appearing during human pregnancy were present. Large tumorous cells, many of which were in mitosis, were conspicuous (Fig. 9). In order to graphically summarize the results of this investigation, Table I is appended.

Discussion. The foregoing observations reveal a possible cause of periarteritis nodosa which hitherto has not been recognized. The evidence points to likely endogenous and etiological factors in the experimental induction of periarteritis in rats. It would appear that the hormonal imbalance developed by estrogen followed by FSH first affects testicular morphology and physiology and may be considered as a "sensitizing" mechanism.

Among the procedures employed by Selye (6,8) which seem most pertinent to this investigation is the production of periarteritis

TABLE I. Graphic Summary of Periarteritis and Associated Phenomena in Experimental Animals.

Mo after treatment autopsied	Reactions in seminiferous tubules	Periarteritis		Associated reactions in various organs
		In testicular vessels	In vessels other than testes	
0	Active mitosis of abnormal spermatocytes	0	0	LTH*
1	*Idem*	0	0	LTH
2	Same reaction in few tubules: Some tubules deteriorating	0	0	LTH; slight adrenal hyperplasia
4	Germinal cells deteriorating: Multinucleate, pyknotic, & vesicular cells	Positive extensive in arterioles of interstitial tissue	0	LTH; pronounced adrenal & pituitary hyperplasia with tumor cells
6	Germinal epithelium degenerated: Fibrotic change in tubules	*Idem*	Present to some extent in mesenteric vessels	*Idem*
8-12	Similar fibrotic change, very extensive	Positive pronounced, very extensive	Very pronounced extensive in mesenteric and other vessels	LTH pronounced: Adrenal and pituitary hyperplasia pronounced with tumor cells

* LTH = Lymphoid tissues hyperplastic.

by the implantation of two 30 mg pellets of the mineralocorticoid desoxycorticosterone acetate (DCA) in rats which had been sensitized by unilateral nephrectomy and given diets rich in sodium and protein. Selye(8) found that administration of adrenocorticotropic hormone (ACTH) stimulates the production of glucocorticoids which in turn alleviate the symptoms of periarteritis. From these results he infers that possibly the anterior pituitary elaborates a hormone under conditions of his experiment which stimulates the production of DCA or DCA-like hormones which are effective in causing periarteritis in conjunction with sensitization and high salt and protein diets.

Periarteritis induced by Selye's treatment had developed at the conclusion of a relatively short 31 day period. In the present experiment it did not develop until long after the conclusion of experimental treatment. Since estrogen and FSH administration has been followed by a demonstrable effect in initiating the testicular changes described in this paper, it may be presumed that they cause the testis to form products which eventually affect the pituitary in the production and release of a corticotropin which in turn stimulates the adrenals to secrete a mineralocorticoid substance (similar at least to DCA) responsible for the delayed appearance of periarteritis in animals described in this report. Evidence for this interpretation is the marked hypertrophy, hyperplasia, and tumorlike alteration in pituitary morphology on the one hand and in the extensive hyperplasia and tumorlike alteration of the adrenals on the other. In this investigation there can be no question of high salt and protein diets playing a significant role in the causation of periarteritis. Its long delayed appearance points rather to slowly developing endogenous factors as causative agents. The accompanying lymphoidal hyperplasia observed in this investigation has not been evaluated. It may be due to the loss of testicular activity.

Summary. 1. The original intention of this investigation was to determine whether FSH would restore estrogen damaged testes of rats to normal. 2. Restoration of normal testicular activity did not occur. Instead fibrotic degeneration of tubules and periarteritis of interstitial arterioles eventually developed. 3. In animals autopsied 8 to 12 months after treatment periarteritis nodosa was present in intestinal mesenteric, pancreatic, splenic, and other small arteries, but not in pulmonary arterioles. Small vessels of kidneys, adrenals,

and pituitary were also involved. 4. Lymphoidal hyperplasia was an accompanying reaction. 5. Eventual testicular degeneration-atrophy, hyperplastic and tumorlike proliferations of the pituitary and adrenal glands seem to result in a delayed and prolonged production of periarteritis very possibly due to a DCA-like adrenal hormone.

The author is indebted to Drs. R. Gaunt and C. H. Sullivan of Ciba Pharmaceutical for Ovocylin and to Dr. S. S. Steelman and Mr. J. Stracke of Armour Laboratories for FSH. Gratitude is extended to my wife, Nancy, for technical assistance and to Dr. J. F. Kuzma, Director of the Department of Pathology, Marquette University, for his encouragement and assistance in interpretation of experimental findings.

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Distribution of Hg^{203}-Labeled Mercaptomerin in Organs of Normal Rabbits.* (21757)

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Mercaptomerin sodium (Thiomerin®) is the only commercially available organic mercurial diuretic agent in which the mercury atom is attached to a sulfur atom. The mercury atom in other such compounds is linked to a carbon atom, and most of these compounds also contain a xanthine drug, which in itself is pharmacologically active. Mercaptomerin sodium, therefore, would seem to be the diuretic of choice for studying the fundamental mechanisms of action of the organically bound mercury atom. Although mercaptomerin sodium has been in clinical use for many years, data concerning its distribution in organs of the body are still meager. This fact is not surprising, since the chemical method for determining trace quantities of mercury in biologic material is difficult and relatively insensitive(1). In a previously reported experiment employing a radioactive isotope of mercury for such a purpose(2), technical difficulties were encountered in the preparation of the samples for beta ray assay, and an unknown amount of mercury was lost through volatilization. These difficulties can now be obviated by gamma counting with a scintillation probe.

The purposes of the present study were (1) to determine the distribution and the concentration of Hg^{203}, administered in therapeutic doses as tagged mercaptomerin sodium, in the organs of normal rabbits; and (2) to determine whether or not a cumulative effect resulted from 2 such doses of this compound.

Material and methods. Mercaptomerin sodium‡ was labeled with Hg^{203} to contain 40 μc

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Hg^{203} was supplied by the Oak Ridge National Laboratory, Oak Ridge, Tenn., on allocation from the U. S. Atomic Energy Commission.

† Established Investigator of American Heart Assn.

‡ We are indebted to Wyeth Institute of Applied Biochemistry for synthesis of Hg^{203}-labeled mercaptomerin sodium.

of Hg^{203} per mg of mercury; each ml of the stock solution contained 40 mg of Hg in 140 mg of mercaptomerin sodium. This product was stored in a frozen state and thawed immediately before dilution with a sterile 0.9% solution of sodium chloride. The saline solution injected into the animals contained 1 mg of Hg per ml. Twelve domestic rabbits of mixed breeds were kept in individual metabolism cages and were fed a stock diet of compressed pellets. Water was given without restriction. Preliminary studies revealed that radioactivity was present in the venous blood of rabbits 24 hours after injection of the tagged material, but was absent at 48 hours. *Group* 1. Each of 6 animals was given one intravenous injection of tagged mercury as mercaptomerin sodium in a dose of 1 mg/kg of body weight. This dosage is equivalent to the amount of mercury contained in 2 ml (80 mg) administered to an 80 kg man. Forty-eight hours later, the animals were killed by air embolism, and the organs were removed for analysis. *Group* 2. Six animals were each given 2 intravenous injections of tagged mercury (1 mg/kg) as mercaptomerin sodium 24 hours apart. The animals were killed by air embolism 48 hours after the second injection. Two 1-2 g aliquots of each organ removed (except the adrenal gland) were placed in 1 dram vials, and the weights of the tissue samples were determined. Each vial was then assayed for gamma ray activity by inserting it in the well of the crystal of a Nuclear-Chicago Model DS-3 scintillation counter which was connected to a Tracerlab Superscaler. A total of 10,000 counts were made on each sample. The results were expressed in terms of the concentration of mercury per gram of wet weight of tissues.

Results. Group 1. The concentrations of mercury found in the various tissues examined have been summarized in Table I. Only the kidney contained an appreciable amount of mercury—(5.71 μg per g, or 2.52% of the injected dose). This was approximately 20 times greater than the concentration found in the liver (0.26 μg per g, or 0.69% of the dose administered), which had the next highest degree of radioactivity. The concentration of mercury in the serum 24 hours after injection was less than 0.01 μg per ml. Eighty-two to 91% of the administered radioactivity was recovered in the urine excreted within 48 hours.

TABLE I. Distribution of Mercury, Administered as Hg^{203}-Labeled Mercaptomerin Sodium, in the Organs of Normal Rabbits.

Dosage = 1 mg mercury/kilo body wt.

Organs	One dose intrav.	2 doses intrav. 24 hr apart
	Conc. of mercury (μg/g wet wt of tissue)	
Kidney	5.71 ±1.94*	9.87 ±4.33*
Liver	.26 ± .14	.48 ± .34
Adrenal	.16 ± .07	.14 ± .18
Appendix	.11 ± .02	.19 ± .08
Spleen	.09 ± .02	.15 ± .13
Lung	.04 ± .01	.18 ± .11
Heart	.02 ± .01	.04 ± .07
Muscle	.01 ± .01	.01 ± .01

* Mean ± stand. dev.

All animals killed 48 hr after last injection of mercaptomerin sodium.

Group 2. The tissue concentrations of mercury following the administration of two doses of labeled mercaptomerin sodium have been summarized in Table I. The mean renal concentration was 9.87 μg per g—approximately 20 times greater than that found in the liver, which had a mean of 0.48 μg per g. Concentrations were considerably lower in other tissues. The mean total renal content of mercury was 3.21% of the administered dose, while the hepatic content was 0.94%.

Comment. Adam(3) has previously reported on the tissue distribution of mercury following its intravenous administration in doses of 1 mg per kg as Hg^{203}-labeled mercuric chloride. The closest comparable data are those from his rabbits sacrificed at 24 hours, while the rabbits in the present study were killed at 48 hours. However, since the biologic half life of mercury bound to tissues is relatively long (on the order of 3 to 4 days) (4), the data from these 2 different sources are comparable as to the relative distribution and concentration of labeled mercuric chloride and labeled mercaptomerin sodium. Twenty-four hours after the administration of the Hg^{203}-labeled mercuric chloride, only 6-15% of the dose had been recovered in the urine. The relative concentrations of mercury in the various organs were similar to those ob-

tained following the injection of the organic mercurial; however, all organs examined contained higher concentrations of mercury than were found in our experiment. The kidney contained 13-22% of the administered dose in a concentration of 23-32 μg per g of tissue; the liver—the organ with the next highest concentration—contained 3-9% of the total dose in concentrations of 0.8-2.9 μg per g.

The greater systemic toxicity of mercuric chloride, as compared with mercaptomerin sodium, can, therefore, be ascribed to the slower renal excretion of mercury and the higher tissue concentrations of this heavy metal. In particular, the concentrations of mercury attained in the kidney following a dose of 1 mg per kg of mercury as mercuric chloride are well above the therapeutic levels.

Data from the present study show that, following the administration of labeled mercaptomerin sodium in a single dose providing 1 mg of Hg per kg of body weight, only the kidney contained an appreciable concentration of mercury. This renal localization of mercury following its administration as mercaptomerin sodium explains the relatively low toxicity of this compound when used in therapeutic amounts.

The administration of 2 therapeutic doses of mercaptomerin sodium 24 hours apart resulted in approximately a 2-fold increase in the renal and hepatic concentration of mercury; these results suggest that frequently repeated administration of this material for therapeutic purposes may cause sufficient accumulation of mercury in these organs to produce toxic symptoms and signs.

Summary. 1. Mercaptomerin sodium tagged with Hg^{203} was administered to 6 normal rabbits in a dosage providing 1 mg of mercury/kilo weight and its tissue distribution was determined 48 hours later. The total renal content was 2.5% of the administered dose of mercury at a concentration of 5.71 μg/g of tissue (wet weight). The next highest concentration and content were found in the liver (0.69% and 0.26 μg per g respectively). Other organs contained negligible concentrations of mercury. 2. In 6 other rabbits, administration of the same dosage of tagged mercaptomerin sodium on two consecutive days resulted 48 hours later in a renal content of 3.21% of the total amount given, at a mean concentration of 9.87 μg/g. The hepatic concentration and content were 0.48 μg/g and 0.94% respectively. Other tissues showed very little cumulative effect.

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Dialyzable Factor in Normal Allantoic Fluid Inhibiting Hemagglutination Patterns with Newcastle Disease Virus.* (21758)

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During experimental procedures requiring tests of the hemagglutinating activity of Newcastle disease virus (NDV), it was noted that chick embryo allantoic fluid suspensions of the strain under observation were inconsistent in their formation of agglutination patterns with chicken erythrocytes. However, dialyzed samples of virus suspensions produced satisfactory results, so it appeared there was a dialyzable inhibiting factor in the allantoic fluid. It has been recognized that some strains of NDV are erratic agglutinators or nonagglutinators of red blood cells. Other workers(1,2) observed that some nonagglutinating allantoic fluid preparations of NDV

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TABLE I. Difference in Hemagglutinating Capacities of Dialyzed and Nondialyzed Suspensions of NDV.

Virus strain	Preparation	Virus dilution 1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256	1-512
Roakin	Dialyzed				4+	4+	4+	4+	4+	—
	Nondialyzed				—	—	—	—	—	—
Nothnick	Dialyzed	3+	3+	3+	4+	3+	—	—	—	—
	Nondialyzed	±	±	±	1+	±	—	—	—	—
Blacksburg	Dialyzed	2+	2+	2+	3+	4+	4+	4+	—	—
	Nondialyzed	2+	2+	2+	3+	4+	4+	4+	—	—

became agglutinating if the virus was sedimented and resuspended in saline, the inhibiting factor remaining in the supernatant allantoic fluid. Cunha *et al.*(2), moreover, showed the inhibiting factor in normal allantoic fluid.

Methods and materials. Virus Preparations. Most of the work was done with the Roakin† strain of NDV. Other strains tested were the Nothnick† and the Blacksburg‡ (vaccine) strains. Fertile eggs were obtained from flocks of New Hampshire red chickens. Virus suspensions were prepared by seeding approximately 10^2 ID_{50} of virus into the allantoic cavities of 11-day embryonated eggs. After 48 hours further incubation, the infectious allantoic fluids were harvested from previously chilled eggs and stored until used. *Dialysis.* Virus suspensions and normal allantoic fluids were dialyzed through cellulose tubing of 0.00072 inch thickness against 100 volumes of buffered (pH 7.2) saline, except when otherwise stated. Flasks containing the dialyzing fluids were kept at 4°C for 20 hours, with occasional manual shaking. *Hemagglutination titrations.* Red blood cells were obtained from adult chickens or chick embryos of 14 days or older. The cells were collected into Alsever's solution and immediately washed three times in buffered saline. Cells were used no later than the day following collection. Virus titrations, prepared by dispensing 0.5 cc amounts of 2 fold saline dilutions of virus suspensions and 0.5 cc of a 1% suspension of red blood cells into tubes 10 × 75 mm with uniformly hemispherical bottoms, were read after 1 hour at 4°C.

† Supplied by Dr. F. R. Beaudette of the New Jersey Agri. Exp. Station, New Brunswick, N. J.

‡ Supplied by Dr. Herald Cox of Lederle Laboratories, Pearl River, N. Y.

Results. Difference in hemagglutinating capacities of dialyzed and nondialyzed preparations of NDV. Hemagglutination titrations were prepared on dialyzed and nondialyzed allantoic fluid suspensions of Roakin, Nothnick and Blacksburg strains. The Roakin and Nothnick titrations were read after 1 hour at 4°C, but the Blacksburg, which does not agglutinate in the cold, was read after 1 hour at room temperature. The results, shown in Table I, indicate the inhibiting effect of the nondialyzed suspensions on the Roakin and Nothnick strains. The Blacksburg strain suffers less effect. The settling of the Blacksburg titration at room temperature may be significant. It will be shown in a later section that the inhibitor is more effective at lower temperatures.

Recovery of inhibiting factor in dialysate of normal allantoic fluid. Dialyzed and undialyzed samples of allantoic fluid from normal 13 day embryos were tested for inhibitor by preparing 0.25 cc amounts of 2 fold dilutions in buffered saline. Each dilution received 0.25 cc amounts of dialyzed virus suspension containing 4 agglutinating units, and 0.5 cc of 1% red blood cells. In like manner, dialysate from a third sample of normal allantoic fluid, dialyzed against an equal volume of buffered saline, was tested for inhibitor. Results, seen in Table II, show undialyzed allantoic fluid contained 16 times as much inhibitor as dialyzed, and demonstrate the presence of inhibitor in the dialysate.

Effect of temperature on the inhibiting activity. To determine the effect of temperature on inhibitory action, 2 titrations of dialysate containing inhibitor were prepared as described above. One titration was prepared with all reagents warmed to 25°C. The other

TABLE II. Presence of Inhibitor in Normal Allantoic Fluid and Dialysate.

Preparation	Dilution 1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256	1-512
Undialyzed normal allantoic fluid	—	—	—	—	—	—	2+	3+	4+
Dialyzed normal allantoic fluid	—	±	2+	4+	4+	4+	4+	4+	4+
Normal allantoic fluid dialysate	—	—	—	—	—	±	4+	4+	4+

was prepared with all reagents, including the titration tubes, chilled in ice water during preparation. Both titrations were kept at 4°C for 1 hour before reading. A typical result is shown in Table III. The inhibitor is more effective when all reagents are kept chilled during preparation of the titration. The inhibitor itself was heat stable, resisting 100°C for 30 minutes.

Effect of RDE on the inhibitor. Receptor destroying enzyme (RDE) was prepared by the method of Hilleman and Werner(3). The RDE, with a titer of 1:512 for chicken erythrocytes was diluted 1:5 in 0.1% calcium saline and mixed with equal parts of dialysate containing inhibitor. A control mixture was prepared the same way except that the RDE was destroyed before mixing by heating for 1 hour at 60°C. Both mixtures were incubated at 37°C overnight. Then the first mixture was heated 1 hour at 60°C to destroy the RDE. Both samples inhibited agglutination of red blood cells to a dilution of 1-32, indicating that RDE did not destroy the dialyzable inhibitor.

Action of the inhibitor. The inhibitor was effective in greater dilution when it was allowed to mix with the red cells for a brief time before addition of the virus, as shown in Table IV. No evidence was found that the inhibitor permanently changes the ability of red cells to form agglutination patterns when mixed with NDV, since various suspensions of red blood cells (0.5%-1.0%) in dialysate containing inhibitor left for various periods of time (up to 18 hours) at both refrigerator and room temperatures, were as agglutinable as ever when washed free of inhibitor and resuspended in saline. Furthermore, the inhibitor removed from such red blood cells retained its inhibiting activity undiminished.

Since inhibitory action was less marked at room temperature, it appeared possible that virus destroys the inhibitor at room temperature. To test this, 2 fold dilutions of inhibitor were mixed with 4 agglutinating units of virus, allowed to stand at 25°C for 2 hours, then cooled to 4°C before adding red cells. The results showed the inhibitory action was not destroyed but actually was effective in somewhat higher dilution than in control mixtures similarly prepared but kept at 4°C during the whole procedure. This enhancement of inhibitory action was surprising and the phenomena will be the subject of further investigation.

Discussion. It is customary to read NDV hemagglutination titrations after 1 hour at 4°C since rapid elution of virus at room temperature often causes agglutination to disappear before the cells have settled(4,5). One wonders if the variability of agglutination of some strains of NDV may be due to variations in the temperature of the reagents during preparation, the inhibitor being more effective if the reagents are cold and less effective if they warm to room temperature during preparation of the titration.

Within the limits of the experimental procedures employed, the dialyzable inhibiting

TABLE III. Effect of Temperature on the Inhibiting Action.

Titration prepared at	Dilution of dialysate containing inhibitor 1-4	1-8	1-16	1-32	1-64	1-128	1-256	1-512
25°C	—	4+	4+	4+	4+	4+	4+	4+
4	—	—	—	—	±	4+	4+	4+

TABLE IV. Showing Increased Inhibitory Action when Red Cells Are Added before Virus.

Order of addition of reagents	Dilution of dialysate containing inhibitor							
	1-32	1-64	1-128	1-256	1-512	1-1024	1-2048	1-4096
Inhibitor, virus red cells	—	±	4+	4+	4+	4+	4+	4+
Inhibitor, red cells, virus	—	—	—	—	±	3+	4+	4+

factor, or factors, showed no capacity for permanently combining with or changing any of the components of the NDV-hemagglutination system studied. In this it differs from the mucoprotein or mucopolysaccharide type inhibitors of hemagglutination, known to occur in normal allantoic fluid(6,7), which compete with the virus receptors on the red blood cells for combination with the virus and which, like the red cell receptors, are destroyed by action of living virus or by RDE. The dialyzable inhibitor may act in such a way that it weakens the bonds of attraction between virus and red cell, perhaps effecting a reversible equilibrium reaction similar to that described by Tamm(8) in the case of Lee virus and cat erythrocytes.

Summary. An inhibitor of hemagglutination present in allantoic fluid suspensions of certain strains of Newcastle disease virus was largely removed by dialysis so that definite and reproducible hemagglutination patterns were obtained with virus suspensions which had previously appeared to be nonagglutinating. The inhibiting factor was present in normal allantoic fluid of 13-day-old eggs and in the dialysate of dialyzed normal allantoic fluids. The inhibiting activity was 8-16 times more effective at 4°C than at 25°C but the inhibitor was resistant to heating at 100°C for 30 minutes. It was not destroyed by receptor destroying enzyme. The inhibitor was 4 times as effective when mixed with the red blood cells before addition of the virus as when the inhibitor and virus were mixed before adding the red cells. Erythrocytes mixed with inhibitor for varying periods regained their agglutinability if washed free of inhibitor. Moreover, the inhibitor removed from such erythrocytes retained its inhibitory titer undiminished. NDV did not destroy or reduce the inhibitory capacity when allowed to mix with inhibitor for 2 hours at 25°C.

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Estimation of Folic Acid with a Thermophilic Bacillus.* (21759)

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Streptococcus faecalis and *Lactobacillus casei* are generally employed for assay of folic acid(1). Both require complicated culture media(2) and have serious limitations in scope(3,4). In surveying the nutritional requirements of thermophilic bacilli we found a strain which responded to p-aminobenzoic acid (PAB), folic acid (PGA), folinic, N^{10}-formyl PGA, pteroyltriglutamic acid, and PGA conjugate, but did not respond to pteroic or p-aminobenzoylglutamic acid. The simplicity of the other exogenous requirements suggested that this organism might serve for a convenient assay of folic acid and of several important related compounds. Such an assay might allow one to trace the fate of these compounds in the animal more accurately than hitherto possible.

Since this organism appears to utilize conjugates, treatment of natural materials with conjugase may be unnecessary and misleadingly high values resulting from extraneous PGA compounds brought in by conjugase preparations are obviated, as well as erroneously low results due to inability to utilize conjugates(5).

Methods. The organism used is *Bacillus coagulans* No. 3084, kindly supplied by the National Canners Association, Washington, D.C. (now available from the A.T.C.C.). The cultural methods followed are in the main those previously described(6,7). The basal medium is given in Table I, where the ingredients are listed in the order of addition, to minimize precipitation. The medium is made up in double strength and distributed in 2.5 ml amount in 10-ml borosilicate flasks provided with aluminum caps. The solutions to be assayed are pipetted into each flask and the final volume is brought to 5 ml with distilled water. A temperature of 55°C suffices for full growth in 18 hours. *Growth* is measured in optical density (O.D.) units with a Welch Densichron. An O.D. of 1.0 corresponds to 0.55 g/liter dried washed bacteria. Stock cultures are maintained on beef extract (any of several brands) 0.5 g and "Polypeptone" (Baltimore Biological Laboratory) 0.5 g; distilled water is added to 100 ml. After incubation at 55°C for 24 hours, they are stored at 4-7°C. Transfers are made at 4-6 week intervals. Transfers at much longer intervals do not show decreased viability. Twenty-four hours before an assay run, cultures are transferred to agar slants, consisting of the basal medium supplemented with 20 mμg/ml PGA. Qualitative results are usually evident in 8 hours.

TABLE I. Basal Medium for *B. coagulans* No. 3084. Grams (unless otherwise specified) in 100 ml of final medium.

Versen-ol	5 mg
Potassium glycerophosphate	.05
$MgSO_4 \cdot 7H_2O$	.05
Ca (as chloride)	2 mg
Mo (as ammonium molybdate)	.1 mg
V (as sulfate)	.01 mg
Sucrose	1.5
Sodium acetate · $3H_2O$	.04
Trans-aconitic acid*	.3
Dihydroxyethylethylenediamine*	.5
Thiamine · HCl	.2 mg
Biotin	.001 mg
Pyridoxamine · $2H_2O$	.02 mg
DL-asparagine · H_2O	.2
"Fe^3 specific"	.01
Metals mix†	1.5 ml
DL-methionine	5.0 mg
DL-threonine	.01
DL-aspartic acid	.05
DL-alanine	.05
Glycine	.05
L-glutamic acid	.08
pH 6.9-7.1	

* Employed solely as pH buffers.

† 1 ml contains: Ethylenediamine-tetraacetic acid 2.5 mg, Zn (as $ZnSO_4 \cdot 7H_2O$) 2.5 mg, Mn (as $MnSO_4 \cdot H_2O$) .5 mg, Fe (as $FeSO_4 \cdot 7H_2O$) 1.0 mg, Cu (as $CuSO_4 \cdot 5H_2O$) .1 mg, Co (as $CoSO_4 \cdot 7H_2O$) .05 mg, B (as H_3BO_3) .02 mg. Concentrations refer to metal ions alone.

Further details on the basal medium can be found in (7).

Results. The sensitive range is from 0.1

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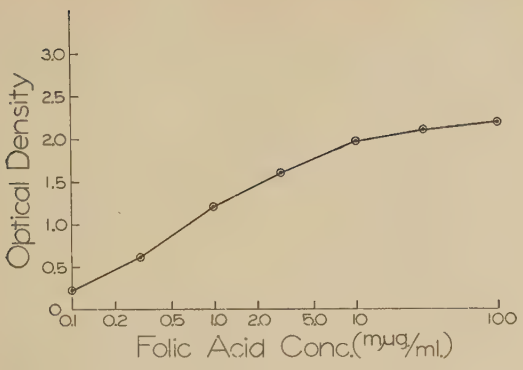

FIG. 1. Response of *B. coagulans* No. 3084 to known concentrations of PGA.

mμg/ml to 10 mμg/ml with PGA as standard (Fig. 1). Above 10 mμg/ml the curve flattens. In the assay of urine interference by PAB is negligible, since PAB is mostly excreted as p-aminohippuric acid(8), to which the organism does not respond (Table II). For materials suspected of containing PAB, the addition of 0.01% sulfanilamide to the basal medium was sufficient to inhibit PAB utilization without affecting the sensitivity towards folic acid.

Five mg PGA was administered orally to 5 control subjects (3 men and 2 women) to gauge the recovery of PGA in urine. Their daily excretion corresponded to 3-5 mμg PGA per ml of urine. In each subject 60-80% of the test dose was recovered in the urine in 10 hours as microbiologically active material. These results are in good agreement with those previously reported(9) in which *Streptococcus faecalis* was used.

Discussion. Judging from the renal clearance tests, the assay provides useful information about the levels of PGA and related compounds in the body; the organism may even permit an unprecedentedly comprehensive estimation of levels of folic acid and related compounds, since it responds to nearly the entire gamut of compounds from PAB to conjugates of PGA. Quite likely, the organism may respond to conjugates of folinic acid or other directly functional forms of PGA. The inability to use p-aminohippuric acid and pteroic acid may be advantageous as these metabolites do not reflect the PGA levels, in contrast to compounds which act as metabolites for this microorganism.

As earlier noted(7), at incubation temperatures of 55°C or higher it is unnecessary to heat-sterilize culture media, as contaminants have not been encountered.

The by-passing of the PAB-folic acid requirement by appropriate combinations of purines, pyrimidines, and amino acids(7) indicates that the folic acid metabolism in this organism is of the type encountered in other bacteria which are auxotrophic for members of the PAB-PGA series(10).

Summary. *Bacillus coagulans* No. 3084, growing optimally at 55°C, has a nutritional requirement which is satisfied by PAB, PGA, folinic, N^{10}-formyl PGA, pteroyltriglutamic

TABLE II. Response of *B. coagulans* No. 3084 to PGA Analogues (Growth Expressed as Optical Density).

	Conc. in mμg/ml	.03	.1	.3	1.	3.	10.	30.	100.
	Molecular wt								
p-aminobenzoic acid	137	.18	.32	.44	.78	1.08	1.5	1.88	2.16
Idem + sulfanilamide .01%	—	.18	.18	.18	.22	.24	.26	.3	.3
p-aminohippuric acid	194	.1	.1	.14	.14	.2	.28	.28	.32
p-aminobenzoyl glutamic acid	266	.2	.38	.38	.38	.4	.42	.5	.58
pteroic acid	330	.12	.12	.15	.2	.23	.23	.4	.4
Folic acid (PGA)	433	.12	.24	.6	1.2	1.6	1.95	2.18	2.28
Folinic acid	461	0	.08	.14	.54	.62	1.3	1.9	2.26
N^{10}-formyl PGA	461	.16	.34	.56	.68	1.0	1.46	1.8	2.16

	Conc. in mμg/ml	1	3	10	30	100	300	1000
Pteroyltriglutamic acid*	691	.12	.14	.42	1.12	2.1	2.26	2.34
PGA conjugate (06433)*	1336	—	—	.14	.28	.52	1.04	1.76

* These compounds are highly unstable, particularly the PGA conjugate (O. D. Bird, personal communication). These results do not take into account the destruction of the compounds due to instability.

acid, PGA conjugate, but not by p-aminobenzoylglutamic acid or p-aminohippuric acid. This organism is unique in its ability to interchange its requirement for PAB with that for PGA. The utilization of PGA conjugates without pretreatment with conjugase renders it valuable for clinical assay. Its nutritional pattern appears well suited for the studies of the folic acid series of metabolites, an expectation corroborated by the results of clearance tests upon human subjects given PGA orally.

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Time Course of Response of Thyroid Gland to Intravenous Injection of Thyrotrophic Hormone. (21760)

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In previous studies dealing with thyroid activity in the rabbit, the "biological decay" of gland radioactivity as determined by *in vivo* neck counting following I^{131} administration was used as a measure of the rate of release of thyroid hormone from the thyroid gland(1). Marked reduction in the rate of release of thyroid hormone was found to begin within two to four hours following thyroxine administration, while exposure to a colder environment led to an acceleration in the rate of thyroid hormone release within 4 hours. Emotional and physical stress was shown to bring about prompt inhibition of I^{131} release(2). Since it is generally accepted that the major factor controlling thyroid activity is the thyroid stimulating hormone (TSH) it was of some interest to compare the time course of these experimentally produced changes in thyroid activity with those following TSH injection. A detailed study of the effects of subcutaneous injections of TSH on I^{131} release in rabbits has been made(1), but it may be misleading to use these findings as a basis of comparison with thyroid responses to physiological stimuli since a TSH preparation from a foreign species was injected and factors related to absorption from

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the injection site, and the behavior of the hormone in the blood may have influenced the latent period and duration of the response. In the following experiments the effects of intravenous injections of purified beef TSH and of rabbit anterior pituitary extracts on I^{131} release from the rabbit thyroid gland have been studied.

Methods. The technic used for measuring thyroid activity in the rabbit as described previously(1) has been modified in that each "neck count" was the mean of eight or twelve individual one minute counts of the neck region, the position of the animal in the clamp being readjusted between each count. The standard error of a "neck count" was between $\pm$ 0.8 and 2.0%. Such counts were made at intervals of ½, 1, 2, 3 and 4 hours after the intravenous injection of either purified beef TSH or rabbit pituitary extract and at intervals over the next 10-15 hours thereafter. A series of counts, within a range of $\pm$ 2%, was obtained over a 2 hour period preceding the injection, and in most experiments, a count within this range was obtained 18 to 24 hours before injection of the TSH preparation. In plotting these data the mean of the control counts has been taken as the 100% level of thyroid radioactivity. Continuous recording of thyroid radioactivity following intravenous TSH injection was obtained by fixing the rabbit in position in the usual type of neck clamp, in a moulded plastic collar carrying a small gamma-sensitive Geiger-Muller tube (G 4 Pb, Twentieth Century Electronics Ltd.) or by fastening the rabbit in the supine position, and suspending a shielded Geiger-Muller tube (G 10 Pb, Twentieth Century Electronics, Ltd.) at a distance of 15 cm from the thyroid gland. The neck region was counted for a 9 minute period in every 10 minutes. Injection of TSH was not made until the count had been stable for at least one hour and in most experiments at least 2 hours. The rabbits were lightly anesthetized with either chloralose-urethane (2.5 ml/kg body weight of a solution containing 2% chloralose and 10% urethane i.v.) or sodium pentobarbital (45 mg/kg body weight i.p.). Endogenous TSH activity in the test animals was suppressed by the intraperitoneal administration of 100 μg of 1-thyroxine (Eltroxin, Glaxo Laboratories Ltd.) in saline suspension at 3-day intervals beginning 48 hours after the administration of the tracer dose of 2 to 10 μc I^{131}(1,3). Purified thyrotrophic hormone (Armour Laboratories, batch No. R-377157) was dissolved in 5.0 ml of 0.9% NaCl solution immediately before injection into a marginal ear vein. All dosages are expressed in terms of the USP reference substance. The pituitary extracts were prepared from female and male rabbit glands within 30 minutes of death. The anterior lobe was dissected free, and prepared for intravenous injection by being ground with sand and 1.0 ml of 0.1 N NaOH solution, the supernatant fluid decanted and taken up to a volume of 10 ml in 0.9% NaCl solution. The extract of a single gland was used for each injection.

Results. In preliminary experiments, a dose of 2.5 mg of TSH administered intravenously was found to be fatal occasionally, whereas 1.0 mg was uniformly well tolerated. A dose of 0.1 mg of TSH induced a discharge of thyroidal radioiodine of up to 19%. Since the average release per day of I^{131} in the normal rabbit is 17%(1) it was felt that this dosage simulated TSH levels encountered normally.

a) Purified beef TSH. Seven rabbits have received a total of 9 intravenous injections of TSH. A typical experiment is illustrated in Fig. 1. One rabbit did not respond to 0.1 mg of TSH, but did respond to 1.0 mg on the following day. An additional rabbit

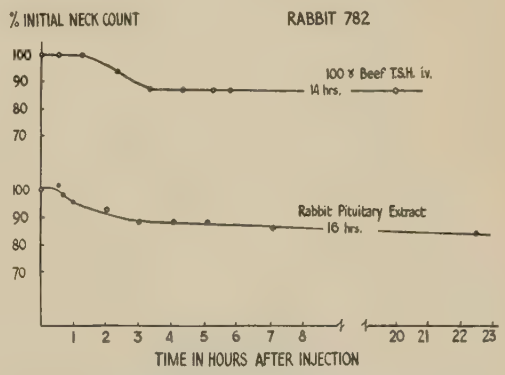

FIG. 1. Comparison of effects of beef TSH and rabbit anterior pituitary extract on thyroid I^{131} content of a thyroxine-treated rabbit.

was given both 0.1 and 1.0 mg doses of TSH. The others were given 0.1 mg. The onset of effect was noted before 60 minutes in 2 experiments, before 120 minutes in a further 3 experiments, before 180 minutes in a further 2 experiments and between 180 and 300 minutes in one experiment. The animals were observed until the release of I^{131} had stopped. It was found that the response following intravenous injection was short-lived in that one-half of the effect had occurred by 80, 80, 130, 140, 150, 180 and 250 minutes respectively following injection, and in 7 out of 8 experiments the effect had worn off by 5 hours following injection. The magnitude of response to the 0.1 mg injection was between 0 and 19% with a mean effect of 10%. The 1.0 mg dose gave responses of 10 and 35% respectively.

b) Rabbit pituitary extract injections: Six rabbits have received a total of 7 intravenous injections of alkaline rabbit anterior pituitary extract, a single gland being used for each injection (Fig. 1). There was a decrease in gland radioactivity beginning before 60 minutes in 2 of the experiments, and before 120 minutes in a further two. In the other 3 experiments the onset of effect was between 110 and 195 minutes. The major part of the effect occurred within 4 hours after injection in 5 out of 6 rabbits. Return to base line rate of release occurred by 5 hours from the time of injection in 6 of 7 experiments. The magnitude of the response varied between 5 and 25% with a mean effect of 13.6%.

There was no qualitative difference in the curves of response in rabbits given purified beef TSH as compared with rabbit pituitary extract in that the duration of the latent period was similar, and the rate of discharge rapidly decreased to control levels between 3 and 5 hours after injection. The shape of the curves obtained was complex, and did not fit a straight line on a semi-logarithmic plot although in several individual experiments, the first 5 hours of response gave a straight line when plotted on log-log paper. In one experiment using beef TSH, and one using rabbit pituitary extract the duration of effect was between 6 and 9 hours. This prolongation of response was probably related to the amount of the TSH injected since the magnitude of response was 32.6% and 25% respectively, which were the largest effects noted.

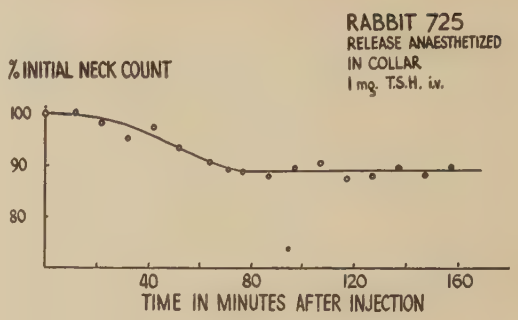

FIG. 2. Effect of intravenous TSH injection on release of I^{131} from thyroid gland of a thyroxine-treated rabbit using a "continuous" counting technic.

c) The time of onset of TSH effect using "continuous" release curves: in order to investigate in more detail the latent period of onset of TSH effect, continuous counting of the thyroid-region for the first 2 hours after TSH injection was carried out, following the intravenous injection of 0.1 to 2.5 mg of purified beef TSH. It is apparent from experiments using discontinuous counting that some rabbits may not respond until after 2 hours, so that in the experiments described here, only quickly reactive glands have been included. Some of the experiments were not completed because of a shift in baseline due to movement of the animal during a count. The results of a typical experiment are shown in Fig. 2. In 7 completed experiments, progressive deviation from the baseline was found to begin before 35 minutes in 3 rabbits, and before 65 minutes in the other 4 rabbits. There was no correlation between the time of onset of effect, and dose given.

Discussion. Following the intravenous injection of purified beef TSH or rabbit pituitary extract, there was, in most rabbits a distinct latent period lasting one-half to two hours preceding the onset of release of I^{131}. A somewhat similar lag period for discharge of labelled thyroid hormone following subcutaneous or intraperitoneal injection has been described in the rat(4), in the chick(5), and in the rabbit(1). Since it is noted even after intravenous injection, the latent period would

not appear to be related to delay in absorption from an injection site. It is more likely that this interval corresponds to the period of activation of the cellular processes which initiate proteolysis and resorption of the stored thyroid hormone. Using cytological methods, DeRobertis(6) has demonstrated the formation of intracytoplasmic colloid droplets as early as 15 minutes after intracardiac injection of TSH in the guinea pig, while resorption of acinar colloid begins by the end of the first hour. During this interval oxidase granules appear in the thyroid cell(7) and an acceleration of cellular metabolism occurs as suggested by an increased P^{32} uptake(8). *In vitro* studies of thyroid slices indicate that oxygen consumption rises within an hour of incubation in a medium containing TSH(9).

The duration of the response to a single intravenous injection of TSH is usually between 2 and 4 hours. In contrast is the more prolonged response noted in rabbits following subcutaneous TSH injection(1). Discharge of I^{131} continued for from 10 to 18 hours in these animals, and over the period of maximum response a roughly exponential curve was obtained. These differences are undoubtedly related to prolongation of absorption from the injection site. An important factor contributing to the brief duration of the decay period of TSH effect is the speed with which the hormone leaves the blood. In the normal rabbit, Loeser(10) demonstrated that thyrotrophic hormone could not be detected in the blood an hour after intravenous injection. Similarly, D'Angelo(11) demonstrated that 99% of the administered dose of TSH had disappeared from the blood in normal rats within 5 hours of intracardiac injection. These workers have used preparations of TSH foreign to the recipient animal, and their observations might have been related to this difference. However, in the experiments reported in this paper, the brief duration of thyroid response is the same for beef and native TSH, and it is therefore likely that the rapid disappearance from the blood, and the rapid falling off of effect on the gland is a characteristic property of the hormone. The presence of corticotrophin in the crude pituitary extract would not be expected to interfere with the response to TSH in view of the demonstration that large doses of cortisone do not influence the response of the thyroid gland to subcutaneous injections of TSH(12).

The results of these experiments demonstrate the rapidity with which changes in the rate of thyroid hormone secretion reflect alterations in blood TSH levels, and are in harmony with the observed time course of thyroid response to cold, thyroxine administration and emotional stress previously described.

Summary. 1. The discharge of organically bound I^{131} from the thyroid gland of the rabbit, as measured by external neck counting, has been used to study the time course of response to intravenous injections of thyrotrophic hormone. 2. Following the intravenous injection of either purified beef TSH or a crude alkaline extract of rabbit anterior pituitary gland there was usually a distinct latent period of between ½ and 2 hours preceding the onset of discharge of I^{131}. 3. The rate of discharge fell off rapidly to approach control levels in less than 5 hours in 13 out of 15 experiments. 4. There are no qualitative differences between the effects of beef or rabbit TSH, indicating that these responses are not species specific.

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Metabolic Interrelationships of Vitamins E and B_6.* (21761)

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Evidence suggesting an interrelationship between vit. E and B_6 has been presented in earlier reports(1,2). The present experiments were designed to further study metabolic interrelationships of these vitamins.

Methods. Weanling Sprague-Dawley rats were divided into 4 groups of 7 to 9 animals each. They were given the same 4 diets previously described(2). The basal diet is deficient in both vit. E and B_6. The 4 groups received this diet without supplement, supplemented with vit. E or B_6 singly, and supplemented with both vit. E and B_6. The rats were placed in metal metabolism cages designed for the collection of urine; body weights and food intake were recorded. The urine was collected under toluene and analyzed for creatine, creatinine, allantoin, and xanthurenic acid. The animals were given intraperitoneal injections of 50 mg of DL-tryptophan per kilo of body weight the day before collecting urine for xanthurenic acid determination. These measurements were made after 70 days of feeding. Creatine and creatinine were determined by the method of Folin(3), allantoin by the method of Young and Conway(4), and xanthurenic acid according to Glazer *et al.*(5). The rats were sacrificed after 110 days of feeding and liver xanthine oxidase activity was measured according to Axelrod and Elvehjem(6).

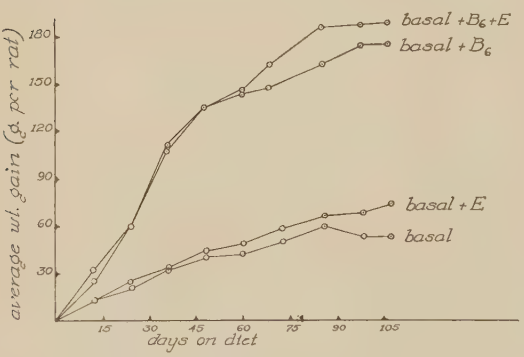

FIG. 1. Influence of dietary vit. E and B_6 on the growth of rats.

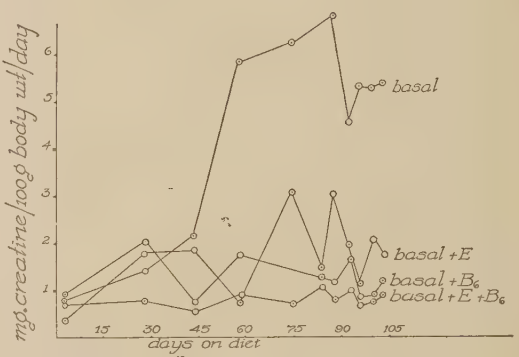

FIG. 2. Influence of dietary vit. E and B_6 on urinary creatine excretion by rats.

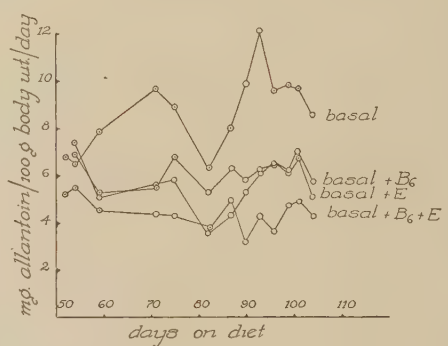

FIG. 3. Influence of dietary vit. E and B_6 on urinary allantoin excretion by rats.

Results. Fig. 1 shows the weight gain of all 4 groups. The inclusion of vit. B_6 in the diet greatly enhanced growth, whereas vit. E had very little effect.

The influence of the various supplements on the excretion of creatine and allantoin is shown by the data presented in Fig. 2 and Fig. 3. Rats receiving the basal diet excreted increased quantities of these metabolites. Supplementation of the diet with either vit. E or B_6 prevented this increase. An elevated

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excretion of creatine and allantoin accompanies vit. E deficiency in the rabbit and these data again emphasize the observation that rats deficient in both vit. E and B_6 behave in a similar manner to rabbits deficient in vit. E (7). Fig. 4 illustrates the xanthurenic acid excretion of all groups, with and without a loading dose of tryptophan. It is quite apparent that the exclusion of B_6 from the diets led to an increased excretion of xanthurenic acid from administered tryptophan. Vit. E had no effect on the excretion of xanthurenic acid.

The liver xanthine oxidase data presented in Fig. 5 are difficult to interpret. Vit. E supplementation tended to reduce the activity of the enyzme and this reduction was most marked when the diet was not supplemented with vit. B_6.

Under the conditions of these experiments, vit. E and B_6 were equally effective in reducing the elevated excretion of allantoin and creatine which developed when the rats were deficient in both vitamins. In contrast, vit. E did not affect the deranged tryptophan metabolism which accompanied vit. B_6 deficiency,

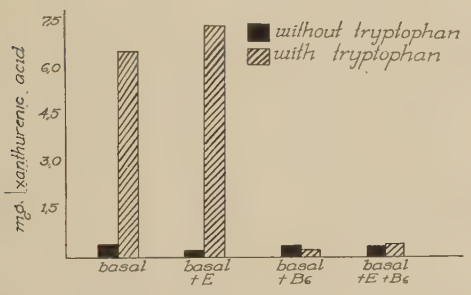

FIG. 4. Influence of dietary vit. E and B_6 on urinary xanthurenic acid excretion by rats with and without administered tryptophan. The data are reported as mg of xanthurenic acid per 100 g body wt per day.

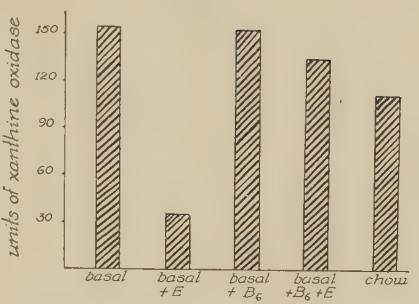

FIG. 5. Influence of dietary vit. E and B_6 on liver xanthine oxidase activity of rats. The units of xanthine oxidase are those described by Axelrod and Elvehjem(6).

and had no effect on growth either in the presence or absence of vit. B_6.

Summary. Rats deficient in both vit. E and B_6 exhibited an increased excretion of allantoin and creatine and supplementation of the diet with either of these vitamins prevented this increase. Vit. B_6 deficiency resulted in a decreased rate of growth and in the excretion of xanthurenic acid following tryptophan administration. Vit. E did not influence growth or xanthurenic acid excretion.

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Transmission of *Trichomonas vaginalis* in the Eye of Animals. (21762)

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Numerous experiments attempting transmission of *Trichomonas vaginalis* (Donne, 1836) to commoner laboratory animals and to many of the more exotic species have failed. The only exception is that *T. vaginalis* has been successfully implanted in the vaginal tract of monkeys(1).

The present report describes experiments in which *T. vaginalis* has been grown in the anterior chamber of the rabbit eye. Preliminary studies on the rat were successful, but the rabbit was found to be the better species because the larger size of the eye and the greater volume of aqueous humor facilitated the work.

Materials and methods. The organism was a strain of *T. vaginalis* isolated January 17, 1955 from a patient with vaginal and urethral trichomoniasis. The original isolation was accomplished on a trypticase medium(2) to which had been added penicillin and streptomycin(3). The culture was transferred 9 times at one to 4 day intervals. It was then seeded into a thioglycollate medium to which had been added horse serum and rice starch (THS medium). The 2-day culture on this THS medium was the material utilized for injection of eyes of Rabbits I and II. All other rabbits were injected with aliquots of the aqueous humor of infected eyes. In making injections or in withdrawing aqueous humor, the cornea was pierced with a No. 26 gauge needle, the tip of which just entered the anterior chamber. For examination of the aqueous humor, 0.1 to 0.2 ml fluid was removed from the anterior chamber. (The rabbit eye contains approximately 0.3 ml of aqueous humor.) This material was examined as a smear under low and high power magnification of the microscope, and cultured in the THS medium for *T. vaginalis*. Both aerobic and anaerobic bacteriologic tests were carried out routinely on each aqueous humor transfer to assure that a pure culture of the parasite was transmitted. (Bacterial contamination occurred only once; this series was terminated.) To obtain sufficient material for smear, the fluid was often diluted either with aqueous humor or with thioglycollate medium. *Smears* were considered positive when *motile* flagellates were visible. Cultures for *T. vaginalis* were always positive when the parasites were visible on smear and were often positive when no parasites could be demonstrated microscopically on smear.

Results. Initial experiment. The anterior chambers of both eyes of Rabbit I were injected with 0.05 ml of the THS culture of *T. vaginalis* already described. Nine days later, smear and culture of the left eye were positive for *T. vaginalis*. On that day, fluid from the left eye was injected directly into the anterior chamber of both eyes of Rabbit IV. Five days afterwards, no organisms were demonstrated by smear or culture of either eye of Rabbit IV. However, 13 days and again 18 days after injection, *T. vaginalis* was demonstrated in the right eye of Rabbit IV, by direct smear and by culture. The left eye failed to show organisms by smear or by culture.

Serial passage in rabbits. Approximately 0.05 ml of the THS culture of *T. vaginalis* was injected into the anterior chamber of both eyes of Rabbit II. Two days later the smear and cultures of the right eye were positive for *T. vaginalis*. The aqueous humor of this eye was used to initiate a continuous series of eye to eye injections which is now in the sixth passage and is being continued (Table I). At intervals of 2 to 7 days, a transfer was made into a new animal. At the time of each transfer, the aqueous humor contained parasites readily visible on smear, and cultures were always positive. At several points in the series, Table I, other chains of passage were instituted and continued without difficulty. A dozen rabbits were injected and examined by smear and culture at frequent intervals. These studies indicated that infection with *T. vaginalis* could be attained consistently al-

TABLE I. Serial Passage in Rabbits.

Date	Rabbit	Inoculum
		ml
4– 4–55	II	.05 culture
	↓	
6	III	.01 aqueous of II*
	↓	
11	V	.01 " " III*
	↓	
15	VII	.02 " " V*
	↓	
22	XI	.01 " " VII*
	↓	
26	XIII	.03 " " XI*
	↓	
5– 2	XV	.02 " " XIII*
	↓	

* Estimate of amount of infected aqueous humor actually injected.

though on occasion some eyes did not become "positive" for many days. Occasionally eyes failed to become infected; this may have been due to inadequacies in technic. The ideal time for transfer appeared to be the fifth day although it was possible to transfer as early as the first day and as late as the fifteenth day.

Pathologic changes in the eye will be reported in detail later. Several days after injection the cornea became opaque, and the fluid when removed from the anterior chamber appeared turbid. At that time parasites appeared in great numbers. A moderate inflammatory exudate was present in the aqueous humor. Sections of the eye disclosed numerous eosinophilic leukocytes and proliferation of the endothelium subjacent to Descemet's membrane with beginning vascularization of the cornea.

Discussion. Survival or growth? The evidence indicating that true growth rather than simple survival has occurred in these experiments is as follows: 1. The parasites have remained alive, motile and apparently as numerous as in the initial inoculum for at least 22 days. 2. On several occasions some time after injection the aqueous humor contained few parasites, or even has been negative on smear or culture, to become strongly positive at a later date, sometimes two weeks afterwards. The possibility that the parasite is in a hitherto undescribed stage of development during the "negative phase" deserves consideration. 3. In the Serial Passage Experiments, the original inoculum was diluted over 1,000,000 times and yet the parasites appeared as numerous as in the aqueous humor of the eye used in the first passage. Studies are being made with inocula consisting of a small counted number of parasites.

Future work. Standardization of this method of infecting eyes with *T. vaginalis* may be helpful in the evaluation of trichomonicidal drugs which hitherto have been studied only *in vitro* or in bacteriologically contaminated tissues. The transmission of other parasites such as *Endamoeba histolytica* or *Giardia lamblia* by this method is being explored.

Summary. Transmission and serial passage of *Trichomonas vaginalis* (Donne, 1836) in the anterior chamber of the rabbit eye is reported.

Addendum. Since this paper was submitted for publication the transmission of *T. vaginalis* has been extended from the sixth to the fourteenth serial passage.

We are grateful to Mrs. Patricia S. Daniels for technical assistance.

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Metabolic Rate Changes Following Thyroid Destruction by AT^{211} and I^{131} in the Rat. (21763)

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The actions of lethal and sublethal doses of astatine^{211} on rats and monkeys as judged by clinical, histological and haematological criteria, as well as studies of the thyroid uptake of At^{211} in man and animals, have been reported(1-5). A myxoedema-like syndrome has been noted in monkeys many months after the administration of At^{211}(1) but no attempt has been made to study precisely the time course of the development of detectable thyroid hormone deficiency or to assess quantitatively its severity.

The present work was undertaken in order to study quantitatively the changes which occur in the overall energy metabolism of rats following a thyroidectomizing dose of At^{211} and to compare these changes with those which occur after a thyroidectomizing dose of iodine^{131}.

Methods and materials. Female Sprague-Dawley rats weighing between 200 and 250 g maintained on a pelleted diet (Purina Chow), with water *ad lib.* and housed in a room having thermostatically controlled heating (75°F.), were used for this investigation. The animals were allowed to become acclimated to the prevailing conditions of diet and temperature for 2 to 3 weeks before the experiment was commenced. The *metabolic rate* of each animal was determined by measuring the time required for the consumption of a measured volume of oxygen from a closed system. The results were expressed as calories per square meter of body surface area per hour, the surface area being calculated from the formula (6):

$$\frac{9 \times \sqrt[3]{\text{Body wt}^2}}{10{,}000} = \text{Body surface area, } (m^2)$$

Basal conditions were approximated by training the animals to the apparatus, by depriving them of food, but not of water, for approximately 20 hours before each measurement; and by making the part of the apparatus in which the rat was placed as small as possible. The results obtained on normal animals by this method are in satisfactory agreement with those published by workers using alternative methods(7). Repeated observations were made on all the animals before the isotopes were administered in order to determine the range of spontaneous variation in the metabolic rate which might be expected under the prevailing laboratory conditions. The *At^{211} was prepared* by a modification of the procedure described by Garrison, *et al.*(8), and was dissolved in isotonic NaCl containing 0.005 g $Na_2SO_3 \cdot 7H_2O$ per ml. Carrier-free I^{131} in the form of NaI was obtained from Oak Ridge National Laboratory and diluted in isotonic NaCl for injection. All of the in-

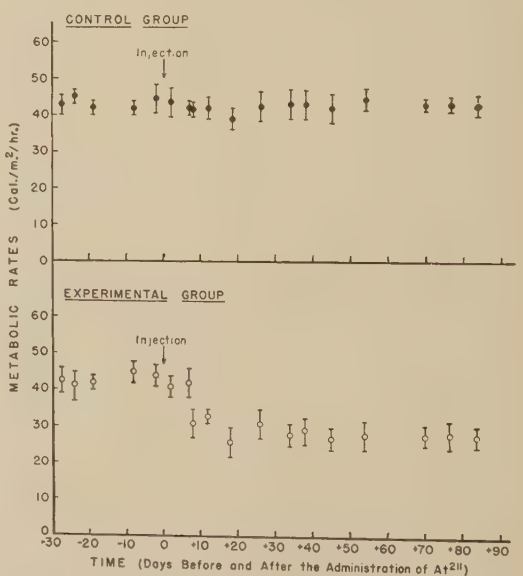

FIG. 1. Metabolic rates of rats given a single intravenous injection of At^{211} (0.8 μc/g body wt) and of control animals given isotonic NaCl. Avg values ± stand. dev. are given for each group of animals.

* Traveling Fellow of the British Postgraduate Medical Federation (University of London) on leave of Absence from Medical College of St. Bartholomew's Hospital, London, England.

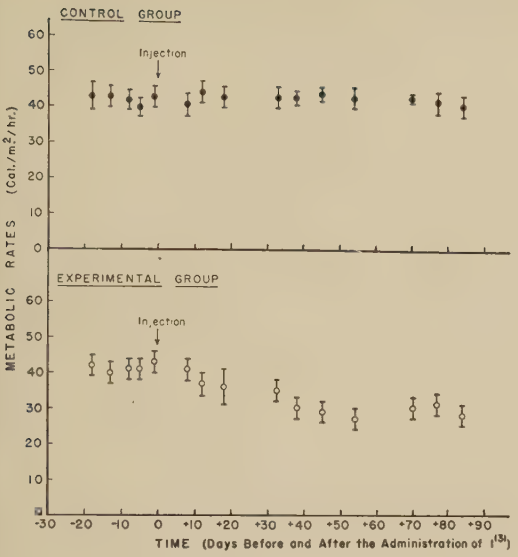

FIG. 2. Metabolic rates of rats given a single intravenous injection of I^{131} (10 μc/g body wt) and of control animals given isotonic NaCl. Avg values $\pm$ stand. dev. are given for each group of animals.

jections were made directly into the surgically exposed external jugular vein under light ether anaesthesia. Twelve rats (average weight 248 g) were given 0.8 μc of At^{211} per g of body weight, and 10 rats (average weight 220 g) were given 10 μc of I^{131} per g of body weight. Two groups of 10 control animals received isotonic saline (0.5 ml per rat).

Results. The average metabolic rates, together with the range plus or minus the Standard Deviation for each group of experimental and control animals are presented in Fig. 1 and 2.

Five of the 12 animals which were given At^{211} died between 8 and 17 days after the administration of the isotope, and, although the metabolic rates of these animals did not differ appreciably from the other members of the group, the mean values and Standard Deviations recorded are based only on observations made on the animals which subsequently survived the whole course of the experiment. The mean metabolic rate of the At^{211}-treated animals decreased sharply to 74% of the control value between 6 and 8 days after the administration of the isotope, and had attained a steady value of between 65 and 70% of the corresponding value for the control group by about 20 days from the date of injection.

None of the I^{131}-injected animals died. The average metabolic rate of this group began to decrease between 8 and 12 days after the administration of I^{131}, attaining a steady value of between 65 and 70% of the control value by about 40 days from the date of injection. The results of an observation made on these animals after 119 days, and not shown in Fig. 2, were also within this range.

Discussion. The doses of At^{211} and I^{131} employed in this study were judged to be sufficient to produce complete radiothyroidectomy. Ideally, it would have been desirable to have used doses of At^{211} and I^{131} which were also comparable from the point of view of their systemic toxicity. This was not possible due to the greater toxicity of At^{211}, the median lethal dose at 60 days (MLD_{60}) for the rat being 1.12 μc of At^{211} per g of body weight and 85 μc of I^{131} per g of body weight (2). The autopsy changes observed in the At^{211}-treated rats which died were similar to those which have been attributed to the effects of internal irradiation by At^{211}(2). The fact that it was not possible to predict from a study of the animals' metabolic rates which members of the At^{211}-treated group would succumb to radiation effects supports the view that the differences observed between the At^{211}-injected and the I^{131}-injected animals were not attributable to the more severe generalized internal irradiation to which the former group was exposed. It has also been shown by Smith, *et al.*(9) that whole body x-irradiation does not affect the rate at which rats consume oxygen (see also(10)).

The radioactive decay of At^{211} (half-life 7.3 hours) and of its radioactive daughter element Po^{211} (half-life 0.52 second) involves mainly the emission of 5.86 Mev. and 7.43 Mev. alpha-particles and an 80 Kev. x-ray. These alpha-particles have a range of about 50 μ in soft tissue. Iodine^{131} (half-life 8.1 days) decays by the emission of negative beta-particles (average energy approximately 0.2 Mev., and range in soft tissue about 2000 μ) plus a cascade of gamma-rays, the majority of which have an average energy of 0.36 Mev.(11).

Thus, for equal numbers of At^{211} and I^{131} atoms *in vivo*, At^{211} results in a greater, but more localized, linear-energy-transfer over a shorter period of time than does I^{131}. It is not possible to estimate the ratio of the numbers of At^{211} and I^{131} atoms present in the thyroid glands of the 2 groups of experimental animals in the present study. However, the dose of At^{211} used was considerably smaller than the dose of I^{131} used; also, a smaller proportion of a given dose of At^{211} as compared to the same dose of I^{131} is taken up by the thyroid gland(1,5). It is unlikely, therefore, that the thyroids of the At^{211}-treated animals actually contained more radioactive atoms than the thyroids of the I^{131}-treated group at any given time. In spite of this, thyroid function, as judged by the animals' metabolic rates, decreased more abruptly in the former than in the latter group, a finding which could be attributed to more acute glandular destruction, *i.e.*, to a greater dissipation of energy within the gland over a relatively short period of time, and hence correlated with the different nuclear properties of At^{211} and I^{131}.

Summary. (1) The time course of the development of hypothyroidism in rats, as judged by the animals' metabolic rates, following the administration of thyroidectomizing doses of At^{211} and I^{131} has been studied. (2) The metabolic rate decreased abruptly to 74% of the control value between 6 and 8 days after the administration of 0.8 μc of At^{211} per g of body weight. A further decrease to the subsequent steady value (between 65% and 70% of the corresponding values for the control group) had occurred by about 20 days from the date of injection. (3) Slowing of the metabolic rate was first observed between 8 and 12 days after the administration of I^{131} (10 μc per g of body weight). The metabolic rate decreased more slowly in the I^{131}-treated than in the At^{211}-treated animals, but ultimately attained a similar steady value(4). It is suggested that the differences which have been observed after the administration of At^{211} and I^{131} may be correlated with the different nuclear properties of the 2 isotopes.

The author is pleased to express his indebtedness to Professor J. G. Hamilton, Director of Crocker Laboratory and to Professor C. W. Asling of the Division of Anatomy of the University of California for provision of the facilities necessary for the prosecution of this work. The At^{211} was prepared by Mr. G. B. Rossi and the staff of the 60-inch Cyclotron. Dr. Patricia W. Durbin and Mr. M. W. Parrott gave generous assistance with the specialized technics involved in the laboratory handling of the At^{211}. The help of all of these colleagues, as well as the skilled secretarial assistance of Miss Catherine Sjoblom, is gratefully acknowledged.

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Serum Properdin Levels in Hemorrhagic Shock.* (21764)

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Recent studies have demonstrated that resistance to bacteria and their toxins is markedly reduced in animals subjected to short periods of hemorrhagic shock(1-4). Among the mechanisms involved in resistance that have been shown to be weakened, are the phagocytosis-promoting factor or factors in serum, and the capacity to neutralize bacterial toxins. Properdin, a serum globulin which acts, in association with complement and magnesium ions, as a natural bactericidal system, is another factor of fundamental importance in resistance(5). Assays of this substance in the serum of dogs before and during hemorrhagic shock were made. This is a preliminary report on the results obtained.

Method. Hemorrhagic shock. Healthy mongrel dogs weighing 15-17 kg were fasted for 12 hours and premedicated with morphine (2 mg/kg). The femoral vessels on one side were exposed under procaine anesthesia and cannulated distal to bulldog clamps to obviate the need for anticoagulants.† After taking a specimen of arterial blood and measuring the femoral arterial pressure directly with a mercury manometer, the dogs were bled rapidly to a mean arterial pressure of 30-40 mm Hg. The pressures were determined intermittently at frequent intervals and maintained in this range by subsequent small hemorrhages or transfusions.‡ The total bleeding volumes ranged from 38 to 58 cc/kg. Three of the animals were kept at 30 to 40 mm Hg until death. Two (P_1 and P_5) were transfused late in shock with a volume of donor blood equal to the total volume bled. Specimens of arterial blood were taken at arbitrary intervals during the course of each experiment. The serum from these specimens was frozen in sealed tubes and maintained at or below −50°C in a "dry-ice" box. When all had been collected they were air-mailed under code to the laboratory of one of us (L. P.) in Cleveland, Ohio, for properdin and total complement (C′) assay(5).

Results. The essential data of each of the 5 experiments performed are given in the accompanying figures.

Four of the 5 animals had normal initial

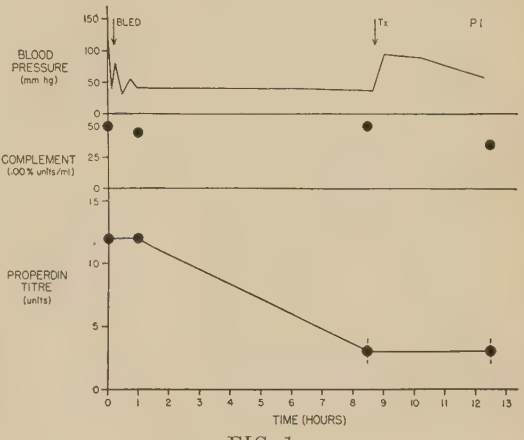

FIG. 1.

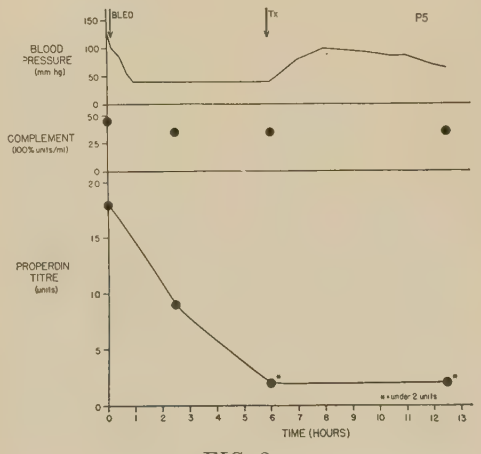

FIG. 2.

* Supported by contract with the Office of the Surgeon General, U. S. Army through the Subcommittee on Shock of the National Research Council.

† The effect of anticoagulants on the assay has not yet been defined, and therefore none were used in these experiments.

‡ All transfused blood was obtained from healthy donor dogs without using anticoagulants.

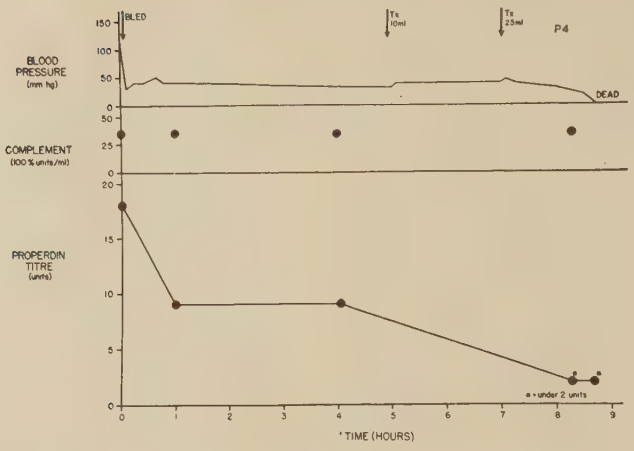

FIG. 3.

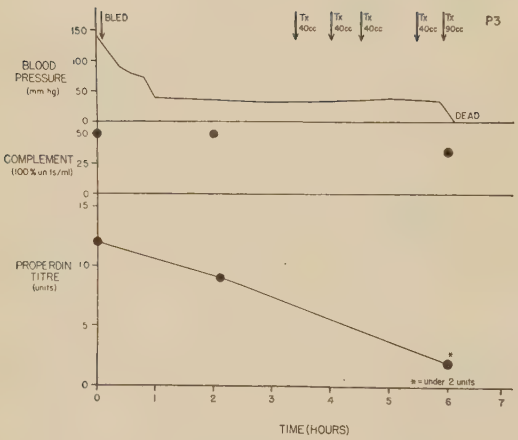

FIG. 4.

properdin titers (12-18 units/ml serum). The fifth (P_2) had a very low initial titer (under 2 units/ml serum). This animal tolerated hypotension very poorly, and died shortly after the mean arterial pressure had been reduced to 30 mm Hg. The tolerance of the other 4 animals to hemorrhagic hypotension was good. Three of these dogs exhibited a distinct reduction of serum properdin within 1-2 hours of the onset of bleeding. All 4 dogs showed a marked reduction of properdin activity later in shock. In 2 dogs (P_1 and P_5), in which the blood volume deficit was corrected late in shock, there was no increase in titer during the temporary pressor response to transfusion.

The total complement assays (100% hemolysis) indicate no significant change in titer except that in several of the experiments a decrease in complement activity occurred as a terminal event.

Comment. This is the first demonstration, to our knowledge, of the reduction of a specific factor of natural resistance in shock. An extended investigation of the role of properdin in hemorrhagic and other forms of shock is in progress.

Summary and conclusions. Properdin levels in the serum of dogs in hemorrhagic shock fall early and progressively. This may be of major importance in the collapse of resistance to bacterial infection which occurs in hemorrhagic shock.

Thanks are due to Leona Wurz for valuable technical assistance.

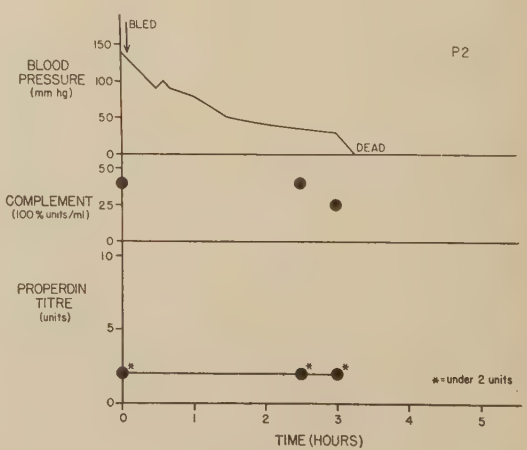

FIG. 5.

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Absorption of Vitamin B_{12} from the Cecum of the Hen.* (21765)

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It has been observed in this laboratory(1), in agreement with published data(2,3), that the vit. B_{12} in the contents of the ceca of the hen amounts to 6-7 μg per g dry matter. These comparatively high B_{12} values are found in the ceca even though the bird is maintained on a B_{12} deficient diet. The further observation, that long periods of time are required to deplete a hen of B_{12} when fed a corn-soybean meal type diet, indicates possible absorption of the vitamin from the ceca.

This problem was studied by determining; (1) the effect of cecectomy on the rate of depletion of B_{12} in the egg, and (2) the absorption of Co-60 labeled vit. B_{12} from the occluded cecum. For purposes of comparison the absorption of S-35 labeled methionine and sodium sulfate from the cecum was studied under the same conditions.

Methods. Rhode Island Red hens were used in these studies. One group of hens was cecectomized. Following the operation, these birds and a control group were maintained on a vit. B_{12} deficient diet(4). The rate of depletion of the vitamin in these birds was followed by estimation of the B_{12} activity of their eggs with *Lactobacillus leichmannii*(5) using cyanide in the extraction procedure. The hens used in the second phase of this study were taken off feed approximately 18 hours prior to injection of the radioactive material. Cecal occlusion was accomplished by inserting a small glass rod (15 mm long) through the cloaca into the rectum, the lower intestine being pushed out through a lateral abdominal incision so that the rod could be directed into the cecum. The glass obstruction was then fixed in place in the cecum near the junction of the intestine by loose ties. Following this, the dose was injected into the lumen of the exposed cecum. Two different doses of Co-60 labeled vit. B_{12} were used, one contained 15 μg with an activity of about 0.6 μc, and the other 4 μg with an activity of about 0.9 μc. Solutions containing 0.75 mg of methionine with an activity of approximately 12 μc or 1 mg of sodium sulfate with an activity of 15 μc were injected. *Blood samples* were taken by heart puncture. The birds were sacrificed at 24 or 48 hours after injection and the various organs and tissues removed for analysis. In a few instances the kidneys were extracted with pH 4.5 acetate buffer in the presence of cyanide and aliquots taken for chromatography. The solvent system used consisted of acetone containing water (5% V/V) and HCl (8% V/V)(6). The chromatograms were developed against "no screen" X-ray film for a period of one month. Using this system B_{12} had an R_f of 0.03 and inorganic cobalt 0.84. Aliquots of the *kidney extracts* and all other samples, after the addition of cobalt or sulfate carrier were subjected to a complete ash procedure

* This project was supported in part by the U. S. Atomic Energy Commission.

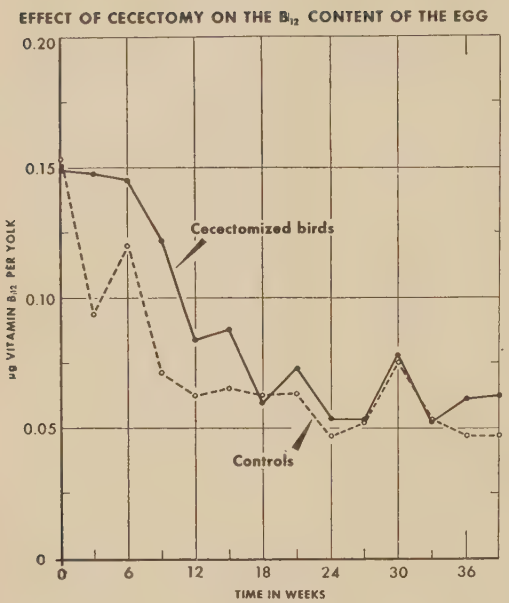

FIG. 1.

using nitric and perchloric acids in order to determine total radioactivity. The solutions remaining after ashing were evaporated to dryness in order to remove the perchloric acid, the salts taken up in concentrated nitric acid and aliquots evaporated and counted in stainless steel cups. Appropriate corrections were made for self-absorption, decay and sampling.

Results and discussion. The curves in Fig. 1 show that there was no difference in the B_{12} content of eggs from cecectomized or normal hens with respect to the severity or the time required for B_{12} depletion. Thus, it appears that the hen on a deficient diet does not derive any appreciable benefit from the B_{12} present in the ceca. This observation is at variance with the statement by Jenkins and coworkers(7) that the chicken possibly derives much of its B_{12} requirement from the ceca. Similarly, our results are not in complete agreement with the interpretation of Shrimpton(8) and Masson(9) who apparently attribute the increase in carcass B_{12} of birds on certain diets to absorption from the ceca. The findings of Jenkins *et al.*(7), that the synthesis of B_{12} occurs throughout the gastrointestinal tract, however, indicates the possibility of absorption of small amounts of intestinally synthesized B_{12} from the tract in general rather than the ceca in particular. This may be an explanation for the long periods of time required to deplete a cecectomized hen of the vitamin.

The results in Table I show the distribution of radioactivity in the organs and tissues examined 24 and 48 hours after injection of Co-60 labeled vit. B_{12}. The average recovery of radioactivity in the cecum was approximately 92% of the administered dose. The radioactivity found in the kidney, liver, and blood indicate that the remainder, *i.e.*, approximately 8% of the dose by difference had been absorbed. Chromatograms of the kidney extracts showed a radioactive spot at an R_f of 0.03 corresponding exactly with that of vit. B_{12}. In no case was a spot observed at the R_f 0.84 corresponding to inorganic cobalt. In addition it was found that 61% of the radioactivity present in the kidney sample was extracted by the butanol procedure used by Davis and Chow(10). These results show definitely that the Co-60 present in the tissues was not in an inorganic form and indicate that it was present as part of the vit. B_{12} molecule.

Since it is generally recognized that B_{12} readily combines with protein materials it is assumed that the comparatively small amount of radioactivity that appeared in the tissues, *i.e.*, approximately 8% of the dose, by difference, was absorbed before the vitamin was bound or otherwise made unavailable for absorption. Had the injected vit. B_{12} been as available for absorption during the entire experimental period as it was initially then the quantity of the vitamin absorbed at 48 hours

TABLE I. Distribution of Radioactivity in Tissues following Injection of Co-60 Labeled Vit. B_{12} into the Occluded Cecum.

Organs and tissues examined	% of inj. radioactivity recovered in individual trials				
	24 hr		48 hr		
	Hen A	Hen B	Hen C	Hen D	Hen E*
Inj. Cecum	90.0	95.9	85.5	96.3	92.1
Liver	.25	.17	—	—	.07
Kidney	.40	.30	—	—	.24
Blood†	—	—	5.2	1.1	2.8

* In addition to the tissues listed, in this trial the uninjected cecum plus the lower gut and the spleen were examined but showed no detectable radioactivity.

† Total blood estimated as 10% of body wt.

TABLE II. Absorption of Vit. B_{12}, Methionine, and Na_2SO_4 from the Occluded Cecum.

Compound inj. into occluded cecum	% of inj. radioactivity recovered				
	Occluded cecum 48 hr	Blood† 2 hr	Blood† 6 hr	Blood† 24 hr	Blood† 48 hr
Vit. B_{12}	91 *	1.2	1.8	2.4	1.1
Methionine‡	6.7	33.1	23.4	9.7	6.9
Na_2SO_4‡	3.5	28.2	24.6	7.6	4.8

* Avg value from trials C, D, and E, Table I.
† Total blood estimated as 10% of body wt.
‡ Avg values from 4 trials.

should have been twice that absorbed at 24 hours. This was apparently not the case, since there was no significant difference in the quantity of vit. B_{12} absorbed from the occluded cecum at 24 or 48 hours.

The results in Table II show practically complete absorption of the sodium sulfate and methionine as compared with the absorption of only 9% of the vit. B_{12} after 48 hours. These data show that there was no interference with absorption under our conditions and support the assumption that the free vit. B_{12} was bound by the cecal contents and thus its absorption was limited.

Summary. 1. Cecectomy did not shorten the time required to deplete the egg of vit. B_{12} when the hen was fed a corn-soybean meal type diet deficient in the vitamin. Thus it appears that the hen on a deficient diet does not derive any appreciable benefit from the B_{12} present in the ceca. 2. Following injection of Co-60 labeled vit. B_{12} into the occluded cecum of the hen, small amounts of radioactivity, chromatographically similar to B_{12} were found in the tissues. The data indicate that this B_{12} was absorbed as the free vitamin and that the B_{12} retained in the cecum became bound. It is suggested that the vit. B_{12} normally present in the ceca of the hen is essentially unavailable for absorption. 3. When S-35 labeled methionine or sodium sulfate was injected into the cecum an average of 93 and 96%, respectively, of the dose was absorbed within 48 hours. Studies on the uptake of these 2 compounds in the blood show that they were rapidly absorbed.

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Protective Effect of Spleen Shielding on Susceptibility of Irradiated Mice to *Escherichia coli.** (21766)

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Shielding of spleen with lead protects the exposed animals from effects of radiation. Similarly, implantation of spleen and injection of spleen or bone marrow homogenates reduces mortality as well as bacteremia in irradiated mice(1-4), and increases resistance of sublethally irradiated mice to certain microorganisms (5,6).

It has been shown by some of us that sublethally irradiated mice are much more susceptible to experimental infection with *Escherichia coli,* an organism indigenous to the mouse(7). The present report deals with the protective effect of spleen shielding during irradiation on susceptibility to this organism. The results, obtained 2 years ago(8), are published at this time to supplement accumulating information dealing with the role of the intact spleen in radiation sickness.

Materials and methods. Eight- to 10-week-old male and female Namru strain mice(9) weighing 15-20 g and taken from our breeding colony were used in the experiments. The animals were irradiated by means of a Keleket deep therapy X-ray machine. The radiation factors were as follows: 200 KVP, 20 ma, filter 0.25 mm Cu and 1.0 mm Al, HVL 0.75 mm Cu, distance from target to skin 75 cm; output approximately 28 r per minute. All X-ray doses were measured in air. All mice were anesthetized with nembutal (1.5 mg per mouse) prior to operation, which consisted of exteriorization of spleen through a lateral abdominal incision, wrapping it in gauze moistened with warm saline and placing it either in a lead or a paraffin (sham) shield. Certain operated and normal animals were exposed to 350 r total body radiation in the manner described by Shechmeister, Bond, and Swift (10), while other operated and normal animals were sham irradiated. The strain of *E. coli* used in the experiments was isolated from the large intestine of a normal mouse. In order to reduce variation, a lyophilized stock of the agent was prepared in individual ampoules. The entire content of a single ampoule was added to 10 ml of broth which, after static incubation for 10-12 hours at 37°C, constituted the first passage. The second passage was prepared by seeding 1 ml of the first passage per 50 ml of nutrient broth and incubating at 37°C for 18 hours. This second serial passage was used in all experiments. Prior to use each culture was tested for purity and its concentration was determined by a modification of the method of Miles and Misra(11). Mice were inoculated intraperitoneally 48 hours after irradiation with 0.2 ml of undiluted culture, which amount corresponded to 1.2×10^8 organisms.

Results. The results are presented in Table I. In every case the duration of the experiment was limited to 10 days after irradiation (8 days after infection), since all deaths resulting from infection of the x-rayed animals occurred during this period. The surgical procedure involved in the exteriorization of spleen did not prove fatal to any of the operated animals (Group 2), nor did any of the operated mice succumb to the challenge dose of *E. coli* (Group 4). Experimental infection of normal animals with the same number of organisms (Group 1) killed 2 out of 35 mice, the animals dying within 3 days after inoculation. Two out of 30 mice died 6-8 days following exposure to 350 r (Group 3). Similar treatment of the operated and either lead shielded (Group 6) or sham-shielded (Group 7) mice resulted in survival of all of the animals. In certain other experiments (not presented in this report), the period of observation was extended to 20 days following irradiation. Approximately 15% of the non-operated irradiated animals succumbed within

* This investigation has been supported, in part, by the U. S. Atomic Energy Commission.

TABLE I. Effect of Shielding of Exteriorized Spleen on Susceptibility of X-rayed Mice to *E. coli* Administered 48 Hours after Irradiation.

Group	Treatment	No. animals	Deaths on indicated days after infection 1	2	3	4	5	6	7	8	Dead/ Total	% dead
1	Infected (I)	35	1		1						2/35	6
2	Operated (Op)	20									0/20	0
3	X-rayed (X)	30						1		1	2/30	7
4	Op I	20									0/20	0
5	X I	40	5	20	2	3		2	2	4	38/40	95
6	Op Sd X	20									0/20	0
7	Op Sm X	20									0/20	0
8	Op Sd X I	29		2		1	1	1			5/29	17
9	Op Sm X I	33	2	2		6	4	5	3		22/33	67

Sd = Spleen exteriorized, kept in a lead shield.
Sm = " " , placed in a paraffin shield.

this time, but no deaths were observed among either the sham or the lead shielded irradiated animals.

Most of the x-rayed mice (38 out of 40, or 95%, Group 5) and of the sham-shielded irradiated animals (22 out of 33 or 67%, Group 9) succumbed to challenge with *E. coli,* however only 5 out of 29 (17%, Group 8) of spleen-shielded-irradiated animals died subsequent to infection. Sixty-six per cent of irradiated-only animals which succumbed to *E. coli* did so within 48 hours after infection (Group 5). A similar pattern of mortality has been observed after irradiated-only animals were inoculated with either heat-killed suspensions of *E. coli*(12) or *Pasteurella pestis,* strain A-1122(13). Increased susceptibility of irradiated animals to certain endotoxins and exotoxins has also been demonstrated(14,15). The short survival time of most of the irradiated-infected animals (less than 48 hours) coupled with the above observations, raises a question as to the possible role of toxemia in early deaths of these animals. This consideration is also of interest in the light of the correlation of survival of experimentally infected-irradiated mice with the concentrations of peripheral granulocytes.

The difference in the survival-time pattern of operated-irradiated-infected mice (Group 9) compared to irradiated-infected mice (Group 5) cannot be explained at this time. The reality of this distribution is supported by lack of deaths in operated-infected and operated-irradiated groups.

The protective effect of spleen shielding results in the increased number of survivors in the irradiated-infected group, and also leads to a different pattern of survival time of the animals treated in this manner (Group 8).

Summary. Shielding of spleen in animals during irradiation protected them from subsequent infection with *E. coli.* Sixty-six per cent of the dying irradiated-infected animals succumbed within 48 hours, while only 18% of the sham-shielded-irradiated-infected animals succumbed within this time. The possible role of toxemia in experimental infection of irradiated animals is discussed.

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Effect of Adrenalectomy on Tryptophan Peroxidase, Adenosine Deaminase, And Arginase Content of Regenerating Rat Liver.* (21767)

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Tryptophan peroxidase is seemingly unique among mammalian enzymes in that its activity in rat liver can be increased 10- to 15-fold *in vivo* by administration of its substrate, tryptophan(1). The activity can be increased to a lesser extent by administering many other substances(2,3), or by X-irradiating the rat(3); however, adrenalectomy prevents the increases caused by everything tested except tryptophan(2) and cortisone or hydrocortisone(4). We were interested in the effects of partial hepatectomy on the concentration of tryptophan peroxidase, and since it seemed probable that the trauma of operation could lead to an increased enzyme activity in the liver remnant because of adrenal stimulation, we thought it necessary to use adrenalectomized as well as normal rats. We have included in this report some data on urea production in partially hepatectomized rats, as well as the effects of adrenalectomy on the adenosine deaminase and arginase activities of resting and regenerating liver.

Methods. Sprague-Dawley female rats were used in all of these experiments. Most of the rats were between 3 and 6 months old. Adrenalectomies were performed 5 days before hepatectomy; the animals were given drinking water containing 1% NaCl, and received intramuscular injections of desoxycorticosterone acetate, 1 mg/kg, on alternate days. Subtotal hepatectomies were performed essentially according to the method of Brues *et al.*(5), and the original mass of liver was estimated from the amount removed(5). Mortality after hepatectomy was high (40%) in adrenalectomized rats, but negligible in the others. *Tryptophan peroxidase* activity was assayed according to the procedure of Knox (2). "Adaptation" to tryptophan was measured 6 hours after intraperitoneal injection of an aqueous suspension of 2 g/kg DL-tryptophan. Adenosine deaminase was estimated by the method of Schneider and Hogeboom(6); tris (hydroxymethyl) aminomethane was used in place of glycylglycine as a buffer. Arginase was measured by the method of Roberts(7) after manganese activation. The surgically removed liver was used as the control for the subsequent regenerating samples in the aforementioned assays. *Urea* production was estimated in nephrectomized rats according to the technic of Engel(8); urea was measured manometrically(9). The intracellular distribution of tryptophan peroxidase was studied by the method of Schneider(10).

Results. Table I shows that the major portion of tryptophan peroxidase activity was in the supernatant fraction, *i.e.*, not sedimentable by forces of 22,000 *g* in 60 minutes. Because of the complex nature of the system (11) the subcellular fractions were assayed both by themselves and in the presence of an unfractionated homogenate. Since the two procedures gave the same results, it appears

* Work performed under the auspices of the U. S. Atomic Energy Commission.

TABLE I. Intracellular Distribution of Tryptophan Peroxidase in Rat Liver.

Fraction	Exp. 1		Exp. 2	
	% of activity	Relative activity per mg N	% of activity	Relative activity per mg N
Homogenate	100	1	100	1
Nuclei (600 g, 10 min., washed twice)	2.7	.25	6.6	.48
Mitochondria (9000 g, 20 min., washed twice)	3.2	.30	2.2†	.07
Microsomes (22,000 g, 60 min.)	12.3*	.61		
Supernatant	74.1	1.78	90.8	1.62
Recovery	92.3		99.6	

* Not washed.
† Mitochondria and microsomes sedimented together, washed twice.

that all essential components of the system were present in the soluble fraction.

The different behavior of tryptophan peroxidase and adenosine deaminase is shown in Fig. 1. The effect of adrenalectomy on adenosine deaminase activity was almost negligible, the difference between the adrenalectomized and non-adrenalectomized rats on the second day being of questionable significance. However, the difference between the adrenalectomized and non-adrenalectomized rats in regard to tryptophan peroxidase was very marked on the first day. On subsequent days, the differences were much less, and it is doubtful whether the apparent increased rate of recovery of activity in adrenalectomized rats is significant. The fact that the tryptophan peroxidase assay values for the control rats after hepatectomy ran consistently below those of the adrenalectomized animals is due to the fact that a slight activation (11%) of the enzyme occurred as a result of ether anesthesia (Table II). This effect of anesthesia did not occur in adrenalectomized rats (Table II). Had we used other rats, sacrificed without anesthesia, for controls, rather than using each rat as its own control, it is obvious that curves 3 and 4 in Fig. 1 would have been much closer together. The anesthesia effect is short-lived, since in a group of sham-operated rats, we found that 24 hours after anesthesia and laparotomy the tryptophan peroxidase values were essentially normal. Not only was the basal tryptophan peroxidase activity markedly reduced after partial hepatectomy, but the maximum activity after tryptophan injection was also drastically lowered (curve 5, Fig. 1). We did not attempt this experiment in adrenalectomized rats because of the high toxicity of tryptophan to such animals(2).

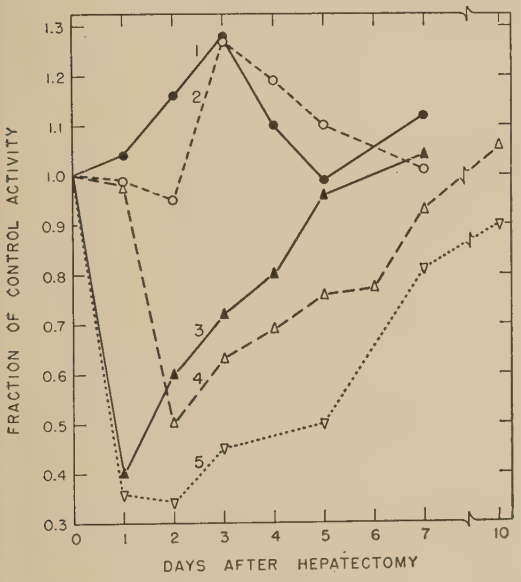

FIG. 1. Effect of adrenalectomy on tryptophan peroxidase and adenosine deaminase activities of regenerating rat liver. The ordinate represents the per cent of the activity of the surgically removed resting liver. Curves 1 and 2, adenosine deaminase: adrenalectomized and non-adrenalectomized respectively. Curves 3-5, tryptophan peroxidase: adrenalectomized, non-adrenalectomized, and non-adrenalectomized 6 hr after 2 g/kg DL-tryptophan, respectively. Three to 8 rats at each point.

Arginase activity measurements showed negligible changes after hepatectomy in either adrenalectomized or control rats. However,

TABLE II. Effect of Adrenalectomy on the Activities of Tryptophan Peroxidase, Adenosine Deaminase, and Arginase in Resting Rat Liver.

Enzyme and units	Control			Adrenalectomized		
	No.	Avg	σ	No.	Avg	σ
Tryptophan peroxidase (μM kynurenine formed/hr/g dry wt)						
Ether anesthesia	44	8.5	2.2	62	7.0	1.1
No anesthesia	54	7.7	1.5	23	7.4	1.9
6 hr after 2 g/kg DL-tryptophan	12	121.3	18.2	—	—	—
Adenosine deaminase (μM adenosine destroyed/hr/g dry wt)	24	52.0	11.6	41	57.9	10.2
Arginase (μM urea formed/hr/mg dry wt)	16	109	15.8	12	83	10.5

adrenalectomy effected a decrease in arginase activity. The values for tryptophan peroxidase, adenosine deaminase, and arginase in resting liver are presented in Table II. The results have been calculated on a dry weight basis; calculation on the basis of total nitrogen would alter somewhat the results during the first 3 days after hepatectomy, but the relative changes would still be the same. Adrenalectomy apparently had no effect on the rate of regeneration of liver tissue, although the per cent dry weight was consistently lower by about 10%.

Fig. 2 shows the rate of urea formation in partially hepatectomized rats, studied over a 24-hour period after bilateral nephrectomy. It is apparent that the capacity of the whole animal to produce urea was impaired; however, the amount of liver remaining was hyperactive, and the rate of urea formation was about 65% greater, one day after hepatectomy, than would be expected solely on the basis of liver size. We could not assess the role of the adrenals in this compensatory effect because of the prohibitively high mortality of adrenalectomized, hepatectomized, nephrectomized rats; less than half of the adrenalectomized rats survived for as long as six hours after bilateral nephrectomy and partial hepatectomy. The effect of adrenalectomy on ureagenesis in rats after nephrectomy alone is well known(8).

Discussion. Studies on enzyme concentrations in regenerating liver have generally shown that activities of most enzymes do not vary markedly from those in resting liver. Among 13 rat liver enzymes studied by Greenstein(12) 48 hours after partial hepatectomy, only xanthine oxidase, which was almost doubled in activity, differed from resting liver values by more than 20%. Novikoff and Potter(13) reported decreases in various mitochondrial respiratory enzymes of 30 to 40% at 15 hours after hepatectomy; this decrease is probably in large part attributable to the decrease in numbers of mitochondria after partial hepatectomy(14). Tsuboi *et al.*(15) noted only slight decreases in activity of four enzymes in regenerating mouse liver.

It was thus of considerable interest to us to find that tryptophan peroxidase was decreased to about 40% of normal 24 hours after par-

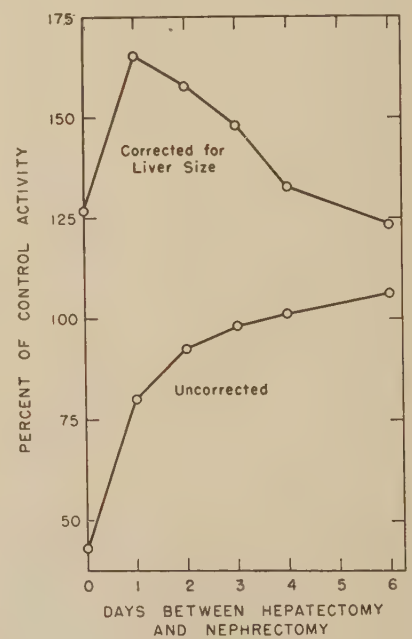

FIG. 2. Urea production in rats after partial hepatectomy. Four to 6 rats at each point. Control value was 1.98 mg urea formed/hr/100 g rat, $\sigma = 0.30$ (42 rats).

tial hepatectomy in adrenalectomized rats. Presumably the mechanisms for controlling both the basal and maximum (*i.e.*, after tryptophan injection) levels of tryptophan peroxidase are impaired during the earlier stages of regeneration, while those controlling other enzymes are not so affected. It was of interest to note that the effect of the adrenals on tryptophan peroxidase activity after hepatectomy was limited only to the first post-operative day, whereas the rate of urea production per unit weight of liver was above normal for at least 3 days after operation.

According to Schneider and Hogeboom(6), adenosine deaminase is a soluble cytoplasmic enzyme. Stern and Mirsky(16), however, showed that nuclei isolated from nonaqueous solvents rather than from sucrose solutions contained appreciable amounts of adenosine deaminase, and proposed that the enzyme was extracted from nuclei in aqueous media. It is entirely possible, of course, that adenosine deaminase occurs in both nuclei and cytoplasm, since Stern and Mirsky did not give complete recovery data. The increase in activity of this enzyme in regenerating liver is what one might expect of a nucleus-associated enzyme, since the nuclei per unit weight of liver tissues also increase to a similar extent in regenerating liver of rats fed *ad libitum*(17). On the other hand, arginase, which is found in both nucleus and cytoplasm(18-20), remained essentially unaltered in concentration throughout the period of hepatic regeneration.

Summary. The concentration of tryptophan peroxidase in the livers of rats remained approximately normal one day after partial hepatectomy, and then decreased to half the control level on the second day. In rats previously adrenalectomized, the activity dropped to a low value on the first day, an indication that in non-adrenalectomized rats the high activity on the first day was attributable to an adrenal adaptive or compensatory response. The maximum tryptophan peroxidase activity, produced by injection of tryptophan, was also markedly reduced after partial hepatectomy. After 7-10 days, the activity was within the normal range. The behavior of adenosine deaminase and arginase in regenerating liver was markedly different from that of tryptophan peroxidase, the former increasing and the latter remaining unchanged. Adrenalectomy decreased the resting value of arginase, but did not alter that of adenosine deaminase.

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Biological Precursors of Biotin and/or Biotin L-Sulfoxide. (21768)

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A number of studies have provided evidence that pimelic acid is a precursor of biotin and/or biotin L-sulfoxide(1-7). During the course of studies involving the recognition, isolation and characterization of biotin L-sulfoxide from aerated cultures of *Aspergillus niger* a number of compounds were studied as possible precursors of biotin L-sulfoxide. While the data are not exhaustive they establish some facts concerning the biosynthesis of biotin and related compounds.

Methods. The biosynthetic experiments were carried out exclusively with *Aspergillus niger*. The mold was grown in 500 ml amounts of medium in 1000-ml Erlenmeyer flasks. The medium had the following composition per liter: sucrose, 30 g; sodium nitrate, 2 g; dipotassium phosphate, 1 g; magnesium sulfate heptahydrate, 0.5 g; potassium chloride, 0.5 g; ferrous sulfate heptahydrate, 0.01 g; supplements tested (except as noted), 1 mg; pH 4.0-4.5. Flasks were incubated at 30°C with shaking at a rate of 200 rpm for 5-7 days. Appropriate dilutions of the culture filtrates were assayed without additional treatment. *Microbiological assays* were performed with a PABA-less strain of *Neurospora crassa*. The basal medium had the following composition per liter: sucrose, 20 g; ammonium tartrate, 5 g; ammonium nitrate, 1 g; potassium dihydrogen phosphate, 1 g; magnesium sulfate heptahydrate, 0.5 g; sodium chloride, 0.1 g; calcium chloride, 0.086 g; trace elements(8), 1 ml; p-aminobenzoic acid, 10 mg. Assays were carried out in 125-ml Erlenmeyer flasks with 25 ml of medium per flask. The extent of growth was measured by weighing the pressed, dried mycelium. Data for a typical experiment where the biotin L-sulfoxide produced by *Aspergillus niger* in media supplemented and unsupplemented with pimelic acid is compared are summarized in Table I.

Results. The effect of carbon chain length on the activity of various dicarboxylic acids as precursors of biotin L-sulfoxide in *Aspergillus niger* is summarized in Fig. 1. As in the experiments of Eakin and Eakin the acids of 6 or less carbon atoms that were studied are inactive. Pimelic and azelaic acids were active as biotin precursors. Suberic and sebacic acids were inactive. Eakin and Eakin reported that in their experiments suberic acid, in addition to pimelic acid and azelaic acid, is qualitatively active but no comparative quantitative data were given. Sebacic acid apparently was not studied. It is suggested that the suberic acid of Eakin and Eakin may have been contaminated with pimelic acid and/or azelaic acid. At least two hypotheses can be advanced to explain the activity of azelaic acid. According to one hypothesis the dicarboxylic acids are subject to competing β-oxidation reactions involving the successive loss of two carbon fragments so that azelaic acid has at least as much chance as pimelic acid of being utilized for biotin synthesis before the competing reac-

TABLE I. Data Demonstrating the Response of *Neurospora crassa* to Biotin and Culture Filtrates of *Aspergillus niger*.

Supplement, γ or ml/25 ml	Mycelium, mg	Biotin L-sulfoxide content (by calculation), γ/ml
0 γ biotin	1.6	
0 "	1.6	
.0005 "	16.6	
.0010 "	25.3	
.0015 "	33.0	
.0020 "	36.0	
.0030 "	44.6	
.0050 "	47.4	
.025 ml sample A*	3.4	
.050 "	4.6	.002
.075 "	6.9	
.125 "	10.5	
.025 ml sample B†	13.3	
.050 "	24.6	
.075 "	33.5	.022
.125 "	46.8	

* Sample A is a culture filtrate from *Aspergillus niger* grown on synthetic medium described.

† Sample B is a culture filtrate from *Aspergillus niger* grown on synthetic medium described but containing in addition 1 mg/l of added pimelic acid.

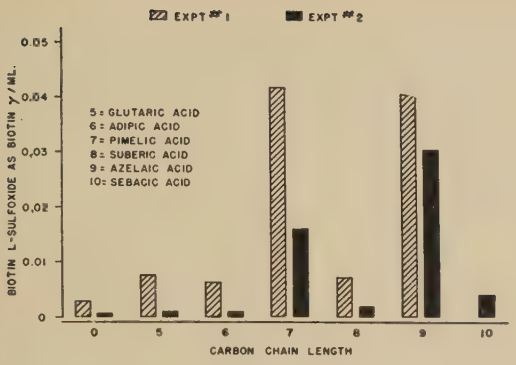

FIG. 1. Effect of carbon chain length on activity of various dicarboxylic acids as precursors of biotin L-sulfoxide.

tions proceed to an extent that the product is useless as a biotin precursor. According to another hypothesis azelaic acid is a precursor because a 9 rather than a 7 carbon compound is actually required to form the biotin molecule. The former hypothesis is favored for a number of reasons. Firstly, azelaic acid is inactive as a source of biotin and/or pimelic acid for the diphtheria bacillus(3). Secondly, an hypothesis involving a competitive β-oxidation where the dicarboxylic acids are subject to repeated loss of two carbon fragments most readily explains the activity of both pimelic acid and azelaic acid (2 carbons too long) and the inactivity of suberic acid (1 carbon too long) and sebacic acid (3 carbons too long).

One postulated function of biotin has been that the vitamin is concerned in the biosynthesis of unsaturated fatty acids. The evidence for this hypothesis is that certain biotin-requiring lactobacilli may be grown on relatively large amounts of unsaturated fatty acids in lieu of biotin(9). The saturated fatty acids are inactive in promoting growth. Since the *in vitro* oxidation of oleic acid, for example, yields azelaic acid as a product, the possibility was considered that the activity of unsaturated fatty acids in promoting growth of the biotin-requiring lactobacilli might be due to the fact that they furnish a potential source of biotin precursor from which limited amounts of biotin are synthesized. In the present experiments both oleic acid and Tween 80 (a polyoxyethylene derivative of sorbitan monooleate) were tested for activity as biotin precursors. Both compounds each in an amount comparable to that of the pimelic acid used are totally inactive.

Similar reasoning suggested that the so-called "traumatic acids"(10) (unsaturated dicarboxylic acids) active as plant growth hormones might function as biotin precursors. The traumatic acids have been shown to be growth factors for certain algea(11). In the present studies 2-decene-1, 10-dicarboxylic acid (yields azelaic acid on oxidative degradation) as well as 1-octene-1, 8-dicarboxylic acid (yields suberic acid) and 1-decene-1, 10-dicarboxylic acid (yields sebacic acid) were tested and found to be inactive as biotin precursors.

The following additional compounds have been tested and found inactive as biotin precursors for *Aspergillus niger*. Lysine, diaminopimelic acid, glutamic acid, α-aminoadipic acid, α-aminopimelic acid, norleucine, ϵ-hydroxy norleucine, α-hydroxy-ϵ-amino-n-caproic acid, ϵ-amino caproic acid, n-hexyl amine, hexamethylene diamine, 1,5-pentanediol, n-amylamine, n-heptanoic acid, n-heptyl amine, and 2-amino heptane.

In contrast to the results of Eakin and Eakin cystine did not augment the effect of pimelic acid. It is believed that this apparent discrepancy may be readily explained. In the experiments of Eakin and Eakin the assays were carried out with *Saccharomyces cerevisiae* which responds to biotin but not to biotin L-sulfoxide(12). In the present studies assays were carried out with *Neurospora crassa* which responds equally well to biotin and biotin L-sulfoxide. Obviously if in the experiment of Eakin and Eakin cystine or cysteine were preferentially oxidized instead of biotin or stabilized the oxidation reduction potential of the medium so that biotin was less readily oxidized enzymatically to the L-sulfoxide these compounds would appear to augment the effect of pimelic acid. Since, as noted above, biotin and biotin L-sulfoxide are equally active for the assay organism *Neurospora crassa* employed here, any effect of cystine or cysteine on the enzymatic oxidation of biotin by the culture would have gone undetected by direct assay with this microorganism.

It follows from the above discussion that cysteine probably is not incorporated as a unit during the biogenesis of biotin as might have been predicted from a consideration of the structures of biotin, pimelic acid and cysteine. The findings are more compatible with the view that the sulfur in biotin is incorporated late in the biogenetic pathway. The activity of desthiobiotin for a number of species (13) as well as the evidence for the microbiological conversion of desthiobiotin to biotin(14,15) are additional arguments supporting this contention.

The following additional compounds each at a level of 5 mg/500 ml of *Aspergillus niger* medium failed to augment the effect of pimelic acid as a precursor of biotin: adenine, adenosine, 3-adenylic acid, 5-adenylic acid, uracil, uridine, guanine, thymine, uridylic acid, cytidine, cytidylic acid, ribose, and orotic acid.

Summary. Pimelic and azelaic acids were found to be precursors of biotin L-sulfoxide in the growth of *Aspergillus niger*. Shorter chain dicarboxylic acids as well as suberic and sebacic acids are inactive. A number of other miscellaneous compounds including oleic acid, Tween 80, and certain "traumatic acids," with structural or functional relationships to biotin and/or pimelic or azelaic acid only in approximate carbon chain length are inactive. A number of other compounds including cystine and certain purine and pyrimidine derivatives do not augment the dicarboxylic acid effect.

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Agglutinins in Sera of Laboratory Workers Exposed to *Serratia marcescens.** (21769)

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Experience with "non-pathogens" has amply demonstrated that under unique circumstances these organisms may give rise to overt or subclinical illness in man. A number of organisms have been incriminated, such as *Neisseria catarrhalis, Aspergillus fumigatus* and *Candida albicans*. Before 1945, *Serratia marcescens* (SM) had rarely been implicated as the cause of human infections(1-4). Paine (5) studied 4 men incidentally exposed to an SM aerosol while testing protective equipment. These persons were exposed for approximately 2½ hours to concentrations of approximately 2,000,000 viable organisms per cu ft of air. Within 4 hours they complained of substernal tightness, cough, lacrimation, myalgia, chills,

* Presented in part at 54th Meeting of the Soc. of Am. Bacteriol., May 1954, Pittsburgh, Pa.

and fever. Two men had agglutinin titers of 1:40 two days after exposure; by the 20th day one titer had risen to 1:80 and the other had fallen to 1:20. The titer of a third man was 1:160 on the second day and 1:40 on the twentieth day. A fourth individual had a titer of 1:80 on the second day and 1:320 on the twentieth day. There was no mention of control titers obtained before exposure to the aerosol.

The most important series of recent illnesses caused by SM was that reported by Wheat, *et al.*(6). They described 11 cases including a fatal endocarditis which was resistant to the use of terramycin, aureomycin, chloramphenicol, streptomycin, penicillin, polymyxin B and sulfonamides. Every illness followed urinary tract instrumentation and occurred in middle aged or elderly individuals with the exception of one 29-year-old man. In 1952 a fatal case of SM septicemia was reported by Patterson, Bannister, and Knight (7). The organism was recovered after the patient had been placed on a chemoprophylactic program prior to surgery. It was resistant to all anti-microbial therapy except for an *in vitro* susceptibility to streptomycin and neomycin. The source of the infection was undetermined. *SM* is frequently used as a simulant for an infectious agent. From time to time many workers in our laboratories have been exposed to aerosols of SM.

Clinical aspects of SM illnesses. Only a few of the many persons at Camp Detrick who are exposed to SM show any signs or symptoms. A review of these cases confirmed Paine's earlier observations. The presenting complaints fell into 3 categories: (1) acute respiratory disease; (2) allergic manifestation, and (3) mild, chronic illness which persists only as long as the person is exposed to contact with the organism by inhalation. Those with acute respiratory disease had complaints of rhinorrhea, conjunctivitis, headache and sore throat. Cough and slight temperature elevation to 99°F have been noted. Nausea, epigastric burning, and diarrhea have occurred infrequently. These persons almost always had pulmonary exposure to clouds of aerosol 4 to 6 hours prior to the onset of clinical symptoms. The organism has been cultured from the pharynx and nasal mucous membrane as late as 48 hours after exposure. The duration of the acute illness was less than 72 hours. A *second group* consisted of those persons who manifest allergic response to SM. Excoriative contact dermatitis, "asthmatic" wheezing with shortness of breath, and conjunctivitis were prevalent in this group. Relief was obtained if the person stayed out of the building housing the agent. Symptoms recurred upon renewal of exposure in the form of direct contact with the organism on the skin or after entrance into a building where an SM aerosol was created. The *third category* was composed of those persons who complained of intermittent fatigue, lassitude, headache, myalgia, and anorexia. No organic abnormalities have been detected. As in the case of the second group, the symptoms disappeared in a few days if the person left the building in which the organism was prevalent. *Several investigators*(8-11) have demonstrated the development of specific agglutinins in animals following the injection of either living or dead SM. In view of these findings it was decided to examine the serums of laboratory personnel for SM agglutinins.

Materials and methods. SM H antigen. An isolation of SM, strain 8UK, was made from a sputum sample of a laboratory technician actively engaged in work with this strain. This organism was highly motile and produced the deep red pigment that is characteristic of SM. Roux flasks of nutrient agar were inoculated with 1 ml of a nutrient broth culture and incubated for 24 hours at 25°C at which time the growth was washed off in physiological saline (0.85%), transferred to a flask containing glass beads and shaken to break up any clumps. An equal volume of 0.6% formalinized saline was added and the suspension was incubated at 37°C for 24 hours and then checked for sterility in thioglycollate broth and on tryptose-phosphate-dextrose agar. This was the stock antigen suspension. Antigen suspensions in 0.3% formalinized saline made up to a No. 3 MacFarland turbidity tube using a Klett-Summerson colorimeter with a red filter were prepared just before making the agglutination tests. *SM antiserum.* Two rabbits were given in-

travenous injections with the stock flagellar antigen (5.3 x 10^{11} organisms per ml). Rabbit no. 8 was injected at 3- to 4-day intervals with 0.3, 0.5, 0.5, and 0.5 ml amounts and Rabbit no. 10 at 7-day intervals with 0.3, 0.5, 0.5, and 0.5 ml amounts. Blood was obtained from the marginal ear vein, the clot was rimmed and the clotted blood was placed in the refrigerator overnight for serum separation. The serum was drawn off by a micropipette and cleared by centrifuging if necessary. All serum was kept at a temperature of –20°C when not in use. No preservative was added. *Agglutination test.* The centrifugation method of Mayer and Dowling(12) was used with modifications in the volumes of the reagents used and in the time of centrifugation. The serum was diluted one to 5 with saline and serial 2-fold dilutions were made from this. Five-tenths ml of antigen was added to 0.5 ml of serum, mixed and then centrifuged at 2000 RPM for 7 minutes in an International No. 2 centrifuge. The tubes were then tapped with the fingers to resuspend the organism. The highest serum dilution capable of agglutinating the antigen was taken as the endpoint. Preliminary investigation showed no difference in titers obtained by this method and by the method of incubating the antigen antiserum mixtures for 1 or 2 hours at 52°C. *Human serum.* Blood samples were obtained from laboratory workers, allowed to clot at room temperature, the clot was then rimmed and the serum was allowed to separate in the refrigerator overnight.

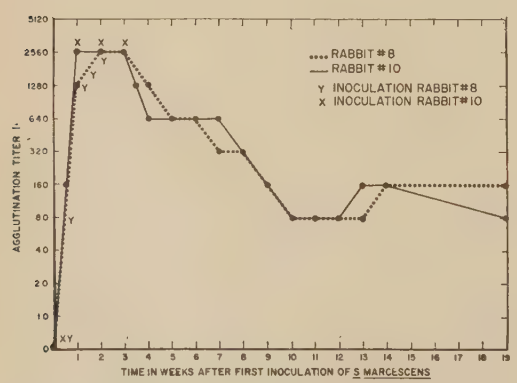

FIG. 1. Agglutination titers of sera of rabbits subjected to injections of *S. marcescens* "H" antigen.

TABLE I. Cross Agglutination Titers of SM "H" Antigen in Heterologous Serums.

Antiserum	Titer
Shigella dysenteriae, polyvalent	1:10
S. paratyphoid B	1:20
B. tularensis	1:20
Br. abortus	0
S. typhosa	0

Serums were siphoned off and kept at –20°C without preservative. The workers were classified under 4 categories according to the type of accidental exposures to SM experienced by the laboratory workers, as follows: Class I—Occasional exposure to low concentrations; Class II—Regular exposure to low concentrations; Class III—Occasional exposure to high concentrations; Class IV—Regular exposure to high concentrations.

Results. Production of antibodies to the flagellar antigen of SM in rabbits, and specificity of antigen. Samples of rabbit sera were collected and tested for agglutinins, before injection with the SM "H" antigen, at various intervals during the course of injections, and at various intervals after completion of the schedule, to determine the approximate initial appearance of antibodies and the titer curve over a period of time. The results are shown graphically in Fig. 1. *Agglutinins* appeared early in the injection series and titers ranging from 1:1280 to 1:2560 were apparent 7 to 14 days after the last injection. The antibody level then dropped steadily to a titer of 1:80, 7 to 8 weeks after the last injection and varied between 1:80 and 1:160 for 9 weeks. To check the *specificity* of the "H" antigen of SM, centrifuge tube agglutination tests were carried out with the following heterologous serums: polyvalent *Shigella dysenteriae, S. paratyphoid B. B. tularensis, Br. abortus,* and *S. typhosa.* Results are shown in Table I. Cross reactions occurred only in low serum dilutions (1:10 to 1:20) and were not considered significant enough to effect the specificity of the test.

Agglutination tests with human serums. Agglutination titers of the serums obtained from laboratory personnel and of samples of pre-employment serums were determined. Samples of pre-employment serums were used as controls. The results shown in Table II

TABLE II. Summary of SM Titers in Laboratory Workers.

Exposure classification*	Total No. of serums tested	Total % of serums having titers of 1: 0†	10	20	40	80	160	320	640	1280	2560
I	43	44	16	19	7	12	2				
II	26	38		19	23	8		4	4		4
III	14	64	14	7		14					
IV	20	15	20	10	20	25		5		5	
Pre-employment	89	79	21								
Total	192	58	17	8	7	7	0.5	1	0.5	0.5	0.5

* I = Occasional exposure to low concentrations of SM.
II = Regular " " " " " " .
III = Occasional " " high " " " .
IV = Regular " " " " " " .
† The lowest dilution tested was 1:10.

were subjected to statistical analysis by the chi-square method and indicate an association between the degree of exposure and antibody titer. Titers were observed more consistently in personnel who were subject to regular exposures to SM (Classes II and IV), than in personnel exposed to occasional exposures (Classes I and III). To obtain a clearer serological picture of the individuals with titers of 1:80 and above, agglutinin titrations were carried out on early serum samples available in the frozen storage bank at the hospital laboratory and on fresh serum samples obtained in December 1952. The results are shown in Table III. In each case where there was only a difference of a few days between the time an individual started employment and the time of the serum sample, no agglutinins were found to be present. Unfortunately, the majority of the serums available were obtained some time after the beginning employment date and agglutinins were already present in varying amounts. However, the general trend was a rise in titer between the first and second serum samples. In general, the titers seem to persist with no change greater than a drop of one, 2-fold dilution.

TABLE III. SM Agglutination Titers Existing in Serum of 21 Laboratory Workers over Extended Periods of Time.

Classification	Date of beginning employment*	Date of 1st available serum sample	Titer of 1st serum sample	May 1952 titer	Dec. 1952 titer
I	2/27/46	4/18/49	1:160	1:80	0
	20	6/16/51	0	"	1:40
	8/ 1/51	†	†	"	"
	2/ 6	†	†	"	†
	16	2/12	1:20	"	†
	4/ 6	6/29	"	1:160	†
II	3/12/51	3/28/51	1:320	1:320	1:320
	5/ 3	10/15	0	1:80	†
	3/28/49	3/ 1/50	1:40	1:2560	1:1280
	8/ 1	3/ 1	"	1:80	†
	2/ 5/51	4/ 4/51	1:20	1:640	1:320
III	11/25/46	†	†	1:80	1:40
	10/ 1/51	†	†	"	"
IV	11/30/51	12/ 3/51	0	1:80	1:80
	2/16	2/13	0	"	†
	1/ 6/52	2/ 4/52	0	"	1:80
	11/10/47	5/23/49	1:80	"	1:10‡
	7/12/49	7/25/51	1:640	"	1:20‡
	5/17/51	10/15	1:80	1:320	†
	12/11/50	2/ 5	0	1:1280	1:640
	3/16/51	8/ 6	0	1:40	1:160

* Date employee actually entered into laboratory work.
† Not available.
‡ 3/10/53.

Development of agglutinins in rabbits exposed to an aerosol of SM. Two rabbits were exposed to an aerosol of SM in a cloud chamber for one hour. Samples of their serums were tested for the appearance of agglutinins to SM before and after exposure. The results to date are given in Table IV. The approximate number of organisms inhaled by each animal was calculated as follows: .3903 = respiratory volume of rabbit/min. in ml/g (13). (cloud conc./ml) (minute vol. in ml/g) (body wt in g) (exposure interval in min.) = no. of organisms inhaled(14). *Rabbit no.* 11 —$(3\text{x}10^2)$ (.3903) (3337) (60) = 2.3 x 10^7 organisms inhaled. Rabbit no. 12—$(3\text{x}10^2)$ (.3903) (3060) = 2.1 x 10^7 organisms inhaled. Rabbit no. 12 developed a titer which was still present 39 days after exposure. Seven days after exposure, serum obtained from this

TABLE IV. SM Agglutinin Titers of Rabbits Exposed* to an Aerosol of SM.

Date	Rabbit No. 11 titer	Rabbit No. 12 titer
1/22/53†	0	0
29	0	0
2/ 2	0	1:320
5	0	1:160
9	0	"
12	0	"
16	1:10	"
24	0	"
3/ 2	0	"

* Exposed on 1/26/53.
† Before exposure.

animal contained enough antibodies to agglutinate SM H antigen in a dilution of 1:320. The titer dropped to 1:160 three days later and was still at this level 25 days later. The other rabbit (no. 11) showed only a transitory antibody titer of 1:10 which appeared on the twenty-first day after exposure.

Discussion. The agglutinin titers for SM found in man suggest that a significant degree of agglutinin formation develops after repeated exposure to SM.

The presence of SM antibodies should not be construed as being diagnostic of specific disease, but it may be interpreted as indicating that such persons are being exposed to excessive concentrations of the organism. The use of protective equipment in such cases seems indicated.

Once an infection with SM is well established in the body, the therapeutic outlook is not promising. Typical of so many "nonpathogens," this simulant is resistant to practically all antibiotics. Penicillin, terramycin, aureomycin, and chloramphenicol are of no value. Colony growth appears to be slowed by sulfadiazine for 1 to 2 days, only to resume its usual growth pattern soon after. Neomycin has been reported as being efficacious. This was established by *in vitro* methods(7). The drug, however, is quite toxic and cannot be used indiscriminately.

Fortunately the majority of illnesses are relatively mild. Recovery is spontaneous within 72 hours except in rare cases of dermatitis.

Summary. 1. Many laboratory workers who inhaled aerosols of SM developed specific serum agglutinins. 2. Agglutinins for SM were found more often and in higher titer in the serums of those who had the most exposure to the organism. 3. Agglutinins for SM tended to persist in man for several months. 4. Rabbits injected intravenously with the flagellar antigen of SM rapidly produced an agglutinin titer of 1:2560 which gradually dropped to a steady level varying between 1:160 and 1:80. 5. One of 2 rabbits exposed to an aerosol of SM rapidly developed agglutinins to a titer of 1:320 which fell to 1:160 three days later and was still at this level 39 days after exposure.

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Concentration of *Teredo* Cellulase.* (21770)

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A cellulytic enzyme system has been demonstrated in woodboring molluscs(1,2). For further studies on the properties of the system, a method of purification or concentration of the cellulase was sought. Previously, such investigations, on other organisms, involved the use of whole tissue extracts and gastric juice, when available(3). A purification of *Myrothecium verrucaria* cellulase was accomplished by Whitaker using fractional precipitation of large quantities of the mold culture (4). However, sufficient amounts of *Teredo* material were not always available, and these were obtained with considerable difficulty at best, since the organisms must be removed whole from the wood which they infect. Lehman(5) has proposed a biochemically specific method for enzyme isolation involving the reversible coupling of an enzyme with a suitable substrate which may be removed from the surrounding medium. The enzyme is subsequently obtained from the substrate by elution with the proper buffer or eluant. The method devised for the concentration of *Teredo* cellulase is based in part on this principle.

Materials and methods. Intact specimens of *Teredo* were obtained from infected wood panels. The digestive tract was removed, weighed, and homogenized in glass with an equal volume of distilled water saturated wth pentachlorophenol. The homogenate was diluted with 5 volumes of distilled water, placed in the refrigerator, and allowed to autolyze for 24 hours. Cellulose substrate was prepared from Hercules CMC Cellulose Gum, Type 70 High. A 0.1% aqueous solution (hot water) containing an amount of cellulose equal to one-tenth the wet weight of the digestive tract material was made up. To this was added gradually and with maximum stirring, one-tenth volume of 10% aqueous $Al_2(SO_4)_3 \cdot 18\ H_2O$. The mixture was centrifuged, the supernatant discarded, and the precipitate washed 3 times with distilled water. Autolyzed gut homogenate was centrifuged to remove particulate material (Fraction A). The supernatant solution was mixed with the packed cellulose, and held at 4°C, with stirring at 15-minute intervals, for 2 hours. Recovery of adsorbed enzyme was accomplished not by elution but by redissolving substrate. The adsorption mixture was first centrifuged at low speed to throw down the cellulose. A fine precipitate of some other material in the homogenate, which flocculated during the adsorption, remained in suspension. The entire supernatant suspension plus one distilled water washing of the cellulose was set aside as Fraction B. In rapid succession the following reagents were then added with stirring to the cellulose: equal volumes of 1N NaOH, 1M Na_2CO_3, and 2 volumes of 1N HCl. Solution then occurred, and the final pH was about 7. The redissolved material was then dialyzed against distilled water for 24 hours. Fractionation of this dialysate was accomplished with $(NH_4)_2SO_4$. Solid salt was first added to 50% saturation. Upon centrifugation, 2 precipitates were observed, one at the bottom of the centrifuge tube (Fraction D), and one at the surface of the supernatant fluid (Fraction C). The latter formed a solid cake and was readily removed with a dissecting needle. The supernatant solution was then made 100% saturated with solid $(NH_4)_2SO_4$,

TABLE I. Fraction Yields from Adsorption Process.

		Exp. 1	Exp. 2	Exp. 3	Exp. 4
Wet wt digestive tract (g)		2.13	1.38	.890	3.01
mg dry wt, fractions A-F	A	86	64	31	85
	B	116	90	47	154
	C	25	10	0	15
	D	15	10	5	16
	E	5	2	0	9
	F	36	20	16	50

* These studies were aided in part by a contract between the Office of Naval Research, Department of the Navy, and the University of Miami, NR 165-271.

TABLE II. Cellulytic Activity of Fractions A-F.

Fraction	Net sugar production as glucose in mg/24 hr			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
A	.027	.050	.020	.039
B	.000	.000	.000	.000
C	.034	.002	.000	.000
D	.002	.026	.000	.005
E	.008	.005	.000	.000
F	1.990	1.024	1.088	3.144

and 2 more fractions were recovered by centrifugation: another surface fraction, E; and one at the bottom of the tube, F. Fractions A-F were all suspended in minimal volumes of water, dialyzed against distilled water overnight, and then lyophilized. Cellulytic activity of the various fractions was assayed by measuring the amount of glucose produced in a medium containing the individual fraction and cellulose. The incubation set-up was similar to that used previously(2), and the amount of sugar produced was evaluated by anthrone reagent(6).

Results. The amount of material recovered in the adsorption process in 4 separate experiments is shown in Table I. For the purpose of measuring the cellulytic activity of the fractions, one-half the dry weight of each fraction was incubated with cellulose. The other half was used as a control and incubated without cellulose, the difference between the two being recorded as Net Sugar Production (Table II). Each incubation mixture had a final volume equivalent to half that of the original digestive tract homogenate. Glucose determinations were done after 24 hours incubation at 25°C.

Cellulytic activity was most apparent in Fraction F; this activity was destroyed on immersing this fraction in a boiling water bath for 30 minutes. In Table III, the activity of Fraction F is compared with 2 samples of whole *Teredo* digestive tract.

TABLE III. Comparison of Activity of Fraction F with Whole *Teredo* Digestive Tract.

Fraction F	mg glucose produced /24 hr	N content of fraction (mg)	mg glucose produced /mg N/24 hr
Exp. 1	1.990	.882	2.26
2	1.024	.490	2.09
3	1.088	.387	2.81
4	3.144	1.23	2.56
Dig. tract	.600	.521	1.15
Idem	.590	.457	1.29

Discussion. Coupled with the advantage of using cellulose as a substrate in these experiments, is the fact that the type used could be reversibly precipitated with little difficulty. The precipitate obtained after treatment with the aluminum salt would not redisperse unless treated with NaOH. A simple neutralization of this basic solution with HCl resulted in reprecipitation, consequently it was necessary to maintain an excess of sodium ion to keep the cellulose dispersed. This was accomplished by the addition of the specified amount of Na_2CO_3. Some of the cellulose was recovered during the ammonium sulfate fractionation as Fractions C and E which conveniently remained on the surface of the supernatant fluid after centrifugation. Fraction F also showed evidence of containing varied amounts of cellulose, but these were all less than 50% of the weight of the total fraction.

It is apparent from the foregoing data that most of the cellulytic activity was present in Fraction F. Autolysis of the homogenized raw material was sufficient to liberate most of the active material into the surrounding liquid. Although some activity was evident in the residue (Fraction A), this was small compared to that of F, and a longer period of autolysis was not attempted.

Dialysis of the various fractions was accomplished using Visking seamless tubing. Although no weakening of the membrane due to cellulytic action was evident under the conditions of the experiment or the duration of dialysis time, the effects of the latter on the fractions, *e.g.* some loss in activity due to adsorption on the membranes, is not yet known.

Summary. A method of concentrating cellulase from marine wood-boring molluscs of the genus *Teredo* has been described. The procedure differs from others in that the cellulase is separated from tissue homogenate by adsorbing it on a precipitated form of cellulose gum. The latter is washed, redispersed, and the cellulase recovered by ammonium sulfate precipitation.

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Cryptococcus neoformans: "An Ascomycete". (21771)

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In a search for the perfect stage of the above named yeast, a mixture of 4 strains of Cryptococci was made on malt extract agar following the method of Wickerham and Burton(1).

Methods. Two strains of *Cryptococcus neoformans* and 2 of *Cryptococcus neoformans var. innocuus* were grown on 3 serial transfers on malt extract agar. Transfers were made daily. On the fourth day, from each of the third transfers, a loopful of cells was placed at the center of a malt extract slant, the 4 piles of cells mixed and spread over the entire surface of the slant. The cultures were incubated at 30°C. Examinations were made at 2, 3, and 4 weeks with no unusual findings.

Results. After 2 months' incubation, a rather unusual type of cell was noted in a nigrosin mount of the mixture of the 4 strains. These cells looked like empty, collapsed sacs with thin transparent walls. Some were attached to normal looking cells, others floated free in the mount. It appeared as though these empty "sacs" had budded out from the normal cells, and we labeled them "ghost" cells (Fig. 1 and 3). Upon further observation, some were noted to contain spores, some 2, others 4 and 8 (Fig. 2, 4, and 5). An examination of the third transfer of each of the 4 pure strains showed that one of the *Cryptococcus neoformans* strains, isolated from a spontaneous infection in a dog, had produced the same sac-like cells, but no spores were noted when first examined. However, they were seen later. Transfers were made both from the malt extract mixture and from the pure dog strain to Noyes' starch ammonium nitrate agar(2). Asci were formed in both instances, a few in one week, many in 3 weeks.

The type of ascus formation appeared quite different from that usually seen in the yeasts, but seemed similar to that described by Lodder and Van Rij(3) for the genus *Lipomyces.* These authors created for this genus a new sub-family of the Endomycetaceae, the Lipomycetoideae, characterized as follows: "Budding cells only, sac-like ascus formed by vegetative cells as a protuberance of the latter; spores oval, light reddish, up to sixteen per ascus. Dissimilation strictly oxidative." There are 2 species listed in this genus separated on the basis of lactose assimilation. They are (1) *Lipomyces lipoferus*—lactose assimilation positive, and (2) *Lipomyces starkeyi*—lactose assimilation negative.

The first of these species was isolated from soil in Holland, and described by Dooren de-Jong(4) as a "New fat producing yeast" which he named *Torula lipofera.* He described an encapsulated yeast but did not find spores. Lodder(5) placed this yeast in the genus *Torulopsis* but later found it to produce ascospores and transferred it to the genus *Lipomyces*(3).

The second species was described more recently by Starkey(6) who had isolated it from soil periodically since 1935. A nitrogen-free agar medium was used in the isolation studies. During the first two weeks Starkey states, "The cells of this yeast consisted entirely of

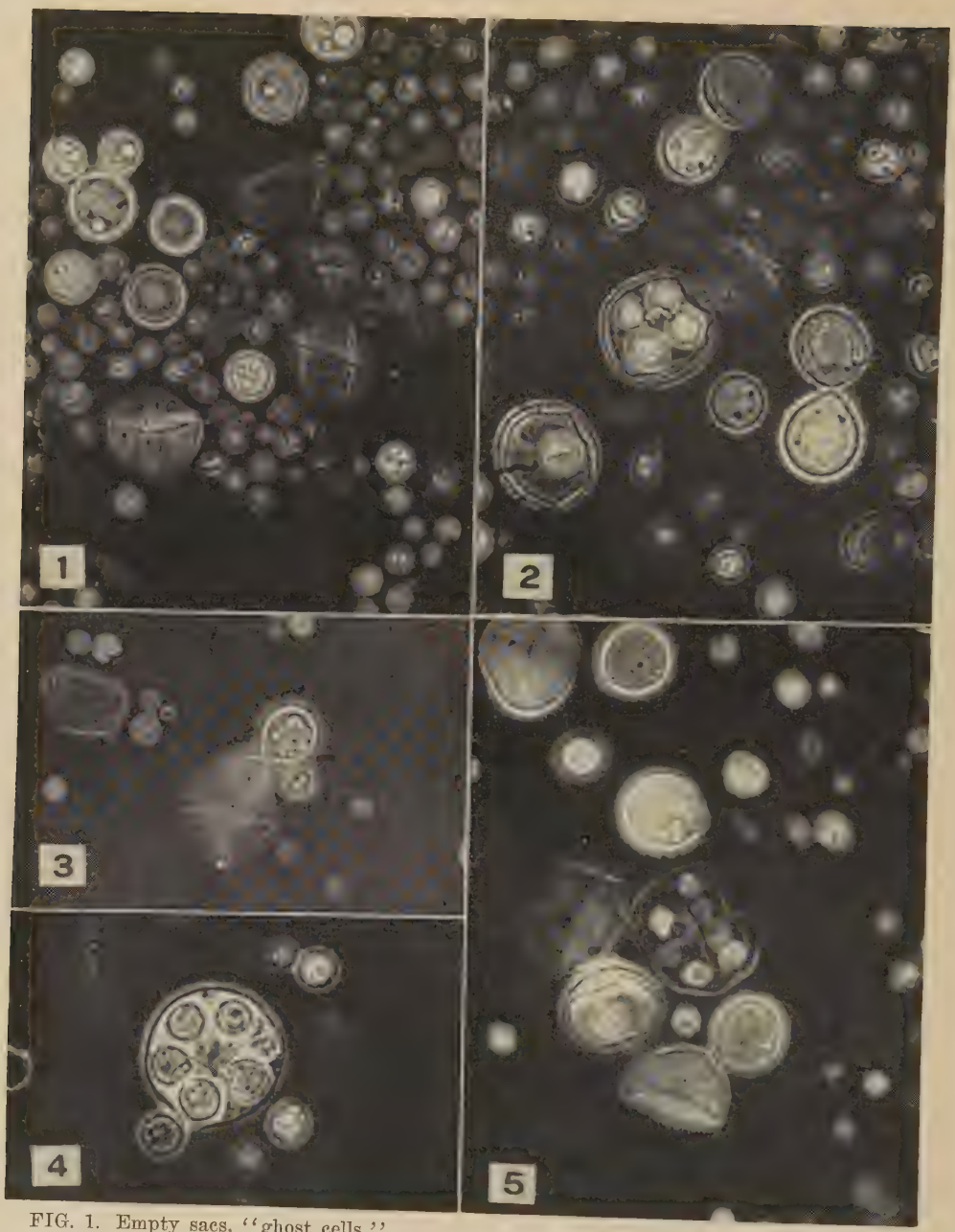

FIG. 1. Empty sacs, "ghost cells."
FIG. 2. Ascus containing 4 spores.
FIG. 3. Empty ascus still attached to mother cell.
FIG. 4. Ascus containing 8 spores.
FIG. 5. Eight spored ascus about to discharge spores.
Nigrosin preparations, magnification ×970.

spherical cells with spherical buds, nearly filled with globules of lipid. During the next few days some of the cells produced buds of a different type. They were shaped like a sac and contained material that had a granular appearance in contrast to the homogeneous refractive lipid globules in the spherical buds. In a few more days the granular substance be-

came transformed into ascospores. The ascus remained attached to the parent cell, even after spore formation was completed. Sometimes a single parent cell bore more than one ascus." Starkey did not find spore formation in pure cultures on the glucose, nitrogen-free agar, but did find them on the vegetable infusion agar of Mrak, Phaff and Douglas(7). They were also produced when heavily inoculated on media unfavorable for growth such as nitrogen-deficient agar media in which rhamose, lactose, ethanol, or glycerol was substituted for glucose. Cells frequently shed their outer membranes, and some pseudomycelia and odd shaped cells were noted. The cells were embedded in slime. The yeast did not ferment glucose nor utilize nitrate nor assimilate lactose. Starkey concluded that the yeast could not be identified as to genus, but placed it in the family Endomycetaceae, subfamily Saccharomycetoideae of the tribe Saccharomyceteae. He states that the yeast in some respects resembles one described by Todd and Herrmann(8) but that the ascospore is different.

Skinner(9) had transferred this yeast to the genus *Cryptococcus* and Lodder and Van Rij(3) while creating a new genus for it, namely *Lipomyces,* call attention to the similarity of it to species now gathered in the genus *Cryptococcus.*

The strain herein described was isolated from a dog brain and identified as *Cryptococcus neoformans,* corresponding in every detail with the criteria cited by Benham(10) as characteristic of this species. It also agrees with the description of Starkey's yeast. However, I believe this to be the first report of this type of ascus formation in a known pathogenic strain of *Cryptococcus.* Emmons(11) has isolated from soil many strains which he identified as *Cryptococcus neoformans* and proved their virulence for mice. We have noted the so-called "ghost" cells in at least one of his strains.

Conclusions. These observations would seem to indicate that *Cryptococcus neoformans* and *Lipomyces starkeyi* may be one and the same organism, and that it is frequently isolated from the soil. Thus another of the fungi pathogenic to man may take its place in that large and important class of the Ascomycetes. It remains but to find ascospores in a strain isolated from a human case. A large number of strains from various sources must be studied and comparisons made both as to virulence and other characteristics. For absolute proof that the structure represents a true ascus one would need to know how the spores are delimited. The final and complete story will be told only by cytological investigations.

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Erythropoietic Action of a Plasma Filtrate in Hypophysectomized Rats. (21772)

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Previous reports(1-3) have described the erythropoietic activity of a filtrate obtained from boiled plasma of rabbits rendered severely anemic by phenylhydrazine. The effects produced in the intact rat by the filtrate were as pronounced as those evoked by anoxic stimuli. In view of the suggestion(1) that the extract might find application in the treatment of refractory anemias in man, it was decided to test its influence upon the anemia of the hypophysectomized rat(4).

Materials and methods. Preparation of the filtrate. Eight adult male rabbits (New Zealand Whites weighing 4-6 kg) were injected subcutaneously on the first day with 25 mg (1 ml) of a neutralized solution of 2.5% phenylhydrazine HCl (Amend Drug & Chemical Co.). From the 2nd to 5th day, the amount injected was increased to from 50-75 mg, in proportion to the weight of the animal. Because of the death of 2 rabbits on the 6th day, it was decided to kill the remaining 6 animals on the 7th day. At this time the rabbits were markedly anemic displaying reticulocyte counts ranging from 76-92%. Blood obtained by cardiac puncture was heparinized (10 mg/50 ml blood), centrifuged for 15 minutes and the pooled plasmas acidified wth 1N HCl to pH 5.5(5,1,2). They were then boiled for 10 minutes. Following filtration, the residue was reboiled in a small amount of acid water (pH 5.5) and refiltered. The 2 filtrates were combined, distributed into 10-ml stoppered vials and stored in a refrigerator (5°C) when not in use. A total of 318 ml of original plasma yielded 110 ml of the final filtrate. *Test hypophysectomized rats.* Pre-treatment counts of peripheral blood cells were made from tail blood of 6 female rats of the Sprague-Dawley strain, hypophysectomized 3½ months previously. They were then injected daily for 10 days with one ml of the plasma filtrate and killed on the 11th day along with 6 untreated hypophysectomized controls.* Determinations were made in these 12 animals of the numbers of formed elements in peripheral blood and bone marrow. Red and white cell counts were made in duplicate. Differential leucocytic percentages were determined from smears stained with Wright's. Chamber counts of eosinophils were made by Randolph's method(6). Reticulocyte values were estimated from dry smears of peripheral blood stained with new methylene blue(7). One thousand erythrocytes were counted and the reticulocytes expressed as a percentage of these. Hemoglobin concentrations were determined by the acid hematin method with a photoelectric colorimeter. Duplicate hematocrit determinations from heparinized blood, spun at 7,000 RPM for 15 minutes, were made by a capillary micromethod. At termination of experiments, all rats were anesthetized lightly with ether and exsanguinated by cardiac puncture. The right femurs were dissected, split lengthwise and the bone marrows removed and placed on a watch glass containing homologous serum. The marrow was prepared in suspension form by drawing it up and down gently with a glass pipette. Smears were then made for determinations of the myelograms. The smears were fixed immediately in absolute methanol for 2 minutes and treated with May-Grünwald stain. A minimum of 1500 nucleated cells was counted for each animal and classified as previously described(8).

Results. The data of Tables I and II indicate clearly that the boiled plasma filtrate evoked a significant erythropoietic response in hypophysectomized rats. Red cell, hemoglobin and hematocrit values were augmented

* It has already been demonstrated(1,2,14) that boiled filtrates prepared from the plasmas of normal untreated rabbits are without effect upon erythropoiesis when tested in rats.

TABLE I. Effects of Plasma Filtrate upon Peripheral Blood Cell Values in Hypophysectomized Rats (Means ± Stand. Errors).

	Untreated hypx rats	Hypx rats given treatment		P values*
		Before treatment	After treatment	
Body wt (g)	108 ± 5.9	113 ± 2.2	110 ± 2.1	ns
RBC (mill./mm³)	5.68± .28	6.32± .26	7.36± .38	<.01
Hb (g/100 ml)	10.7 ± .41	11.3 ± .41	13.6 ± .56	<.05
Hematocrit (%)	35.0 ± 1.54	38.8 ± 1.28	44.6 ± 2.45	<.02
Retics (%)	1.34± .22	1.70± .30	7.8 ± .42	<.01
WBC (thous./mm³)	20.6 ± 2.86	22.2 ± 2.35	20.4 ± 2.44	ns
Eosin (#s/mm³)	710 ±189	764 ±315	728 ±208	ns
Differentials (%)				
Neutrophils	8.8 ± 2.1	8.8 ± 1.2	10.6 ± 2.09	ns
Eosinophils	2.6 ± .67	4.0 ± 1.64	2.8 ± .86	ns
Monocytes	.8 ± .48	.6 ± .24	.8 ± .28	ns
Lymphocytes	87.8 ± 2.15	86.6 ± 2.22	85.8 ± 2.51	ns

* P values calculated from the distribution of Fisher's t. Values of <.05 are considered significant; ns—not significant.

approximately 15-20% while reticulocytes displayed more than a 300% increase. Striking changes were noted in the bone marrows of the treated rats. Whereas the marrows of untreated long-term hypophysectomized rats are usually pale and fatty in appearance, those of the injected animals were grossly reddened. This was due most likely to the erythroid cell hyperplasia involving a doubling in the percentages of nucleated erythrocytes within the femoral marrow.

The various peripheral white cell parameters examined were unaffected by the plasma filtrate. Within the bone marrows, however, significant decreases were observed in the percentages of neutrophilic myeloid elements. None of the other leucocytic values of the myelogram was altered by the experimental treatment.

TABLE II. Effects of Plasma Filtrate upon Myelograms of Hypophysectomized Rats (Mean % ± Stand. Errors).

	Untreated hypx rats	Hypx rats given treatment	P values*
Immat. nuc. RBC	3.4 ± .60	4.4 ± .92	ns
Mature nuc. RBC	19.3 ±2.89	39.1 ±2.22	<.01
Total nuc. RBC	22.7 ±3.33	43.5 ±2.50	<.01
Lymphocytes	15.1 ±3.11	14.2 ±4.64	ns
Immat. neuts.	17.7 ±2.84	10.0 ±1.14	<.01
Mature "	34.4 ±2.89	21.4 ±1.47	<.01
Immat. eosin.	.37± .36	.66± .38	ns
Mature "	8.37±1.12	9.40±1.22	ns
Blasts.	.07± .04	none	—
Misc.	2.33± .64	.80± .45	ns

* P values calculated from distribution of Fisher's t. Values of <.05 are considered significant; ns—not significant.

Discussion. There can be no doubt that the plasma filtrate obtained from rabbits made intensely anemic with phenylhydrazine is strongly erythropoietic not only in intact animals(1-3) but in hypophysectomized rats as well (this report). It should be pointed out that the anemia displayed by the hypophysectomized animal has thus far been ameliorated only by endocrine replacement treatment(4), by lowered barometric pressures(9) and by inorganic cobalt(10). Other agents including iron, copper, folic acid, vit. B_1, B_2, B_6, B_{12}, C, pantothenic acid, liver extracts and protein hydrolysates(11-13), given singly or in some combinations, have exerted little or no effect in overcoming or preventing the post-hypophysectomy anemia.

The present results lend further support to the concept of a circulating "erythropoietin" in animals subjected to various forms of anoxia. The site of formation and mechanism of action† of this factor are problems now un-

† The boiled filtrate showed a negative osazone reaction. This point coupled with the finding that boiled plasma filtrates obtained from chronically-bled rabbits(14) are also effective would render unlikely the possibility that the erythropoietic effects observed in the present experiments are the result of some action specific to the phenylhydrazine itself.

der investigation.

Summary. A filtrate of boiled plasma obtained from rabbits rendered severely anemic by phenylhydrazine was administered daily for 10 days to hypophysectomized rats. Significant increases occurred in peripheral red cell, hemoglobin, hematocrit and reticulocyte values. The bone marrows of these animals showed signs of intense erythroid cell hyperplasia.

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Resilience of the Fetal Eye Following Radiation Insult. (21773)

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There is always a latent period before any physiological or morphological manifestation of the radiation insult. The present study is made on an actively differentiating embryonic organ, namely, the eye of the 13.5 day mouse fetus, examined at 4, 24, and 72 hours and 6 days following x-irradiation to 300 r.

Materials and method. Mature CF_1 female mice were time-mated to males of the same strain and 13.5 days later the pregnant mice were exposed to whole body x-radiations of 300 r. The radiation facilities included a Quadrocondex constant potential therapy machine run at 210 KVP, 15 MA, and in conjunction with 0.31 mm Cu and 0.50 mm Al filters. The output of the machine, as measured in air within the container used to house the mouse, and at a distance of 50 cm to the gravid uterus, was 96.6 r/min. Following exposure the mice were returned to their cages with abundant water and food. At intervals of 4, 24, and 72 hours and 6 days following x-irradiation, pairs of pregnant mice were sacrificed, the lowermost fetuses within the uterine horns were decapitated and the heads were fixed in Bouin's fluid for histological study. Sections were cut at 10μ and mounted serially, and were stained with Delafield's haematoxylin.

Observations. The eye of the 13.5 day fetal mouse is differentiated into a 2-layered cup with distinct retinal primordium and an outer pigmented layer. The lens is separate and well defined. The corneal epithelium is thick, and the vitreous chamber is filled with large and loose mesenchymatous cells. Mitoses are in evidence, particularly in the outer nuclear zone of the retina where the rods will eventually differentiate. Since the retina appears to be quite uniform (homogeneous) there is at this time no differentiation into the various layers of the functional eye (Fig. 1 and 2).

If this eye is exposed to 300 r x-rays and is examined some 4 hours later it is immediately evident that the presumptive retina has

* This paper is based on work performed under Contract for the Atomic Energy Commission.

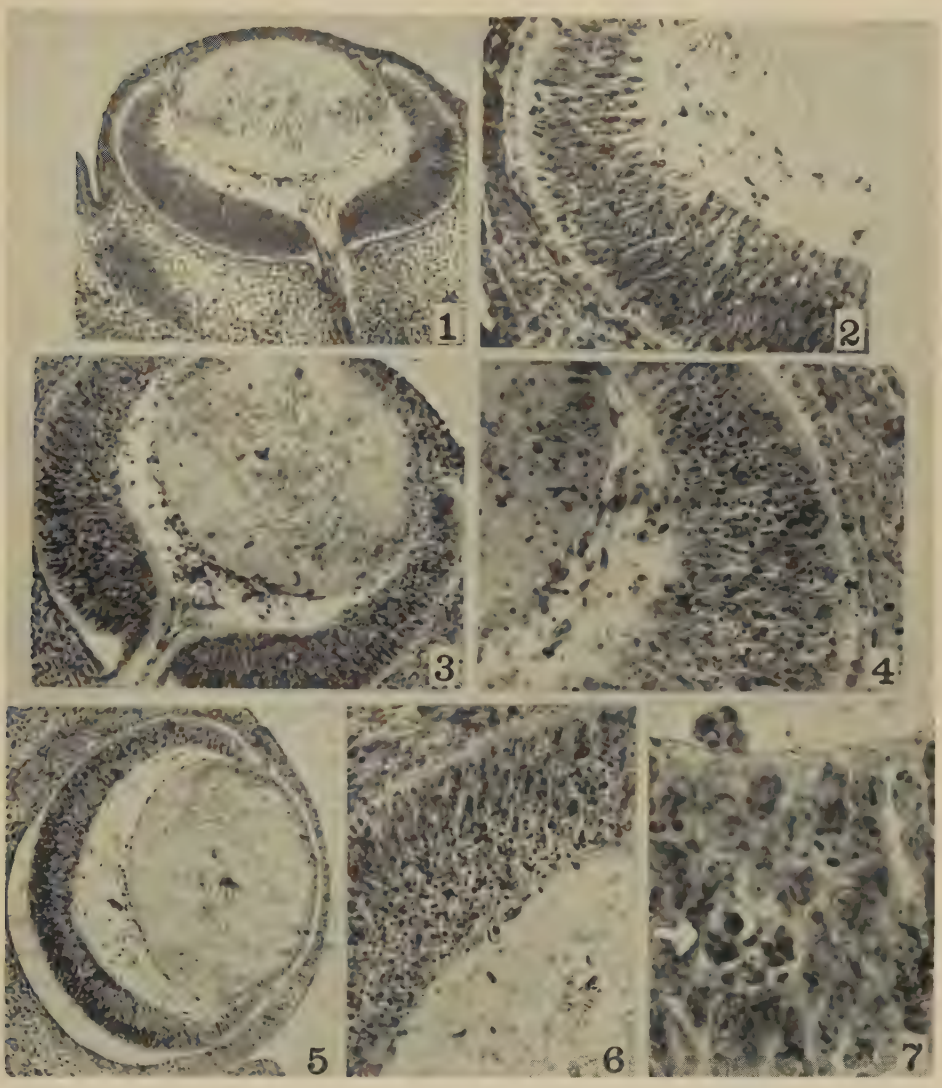

FIG. 1, 2. Normal eye of mouse fetus at 13.5 days. Note abundant mesenchymatous primordium of vitreous body and active mitosis in outer zone of developing retina, where rods will differentiate.

FIG. 3, 4. Fetal eyes exposed at 13.5 days to 300 r whole body x-irradiation of pregnant mouse, and fixed for study 4 hr later. Note cellular debris mixed with primordial cells of vitreous body, and pycnotic nuclei throughout retina, in both inner and outer zones. Pigmented layer and lens apparently unaffected.

FIG. 5, 6, 7. Fetal eyes exposed at 13.5 days to 300 r x-rays and fixed for study 24 hr later. Note widespread destruction of cells, particularly toward the inner zone (Fig. 6) of developing retina. Cellular debris somewhat less in vitreous body. Active phagocytosis within presumptive retina (Fig. 7).

sustained extreme damage to its cellular constituents. The general contour of the retina remains intact but throughout its cellular components are many pycnotic nuclei, fragmented chromatin masses, and dead cells (Fig. 3 and 4). Some of the fragmented nuclei are seen within the vitreous body, having been sloughed off from the retina. If one could estimate the number of nuclei affected it would approach the 50% level, particularly in the vicinity of the optic nerve. The cells near the potential ora serrata appear to

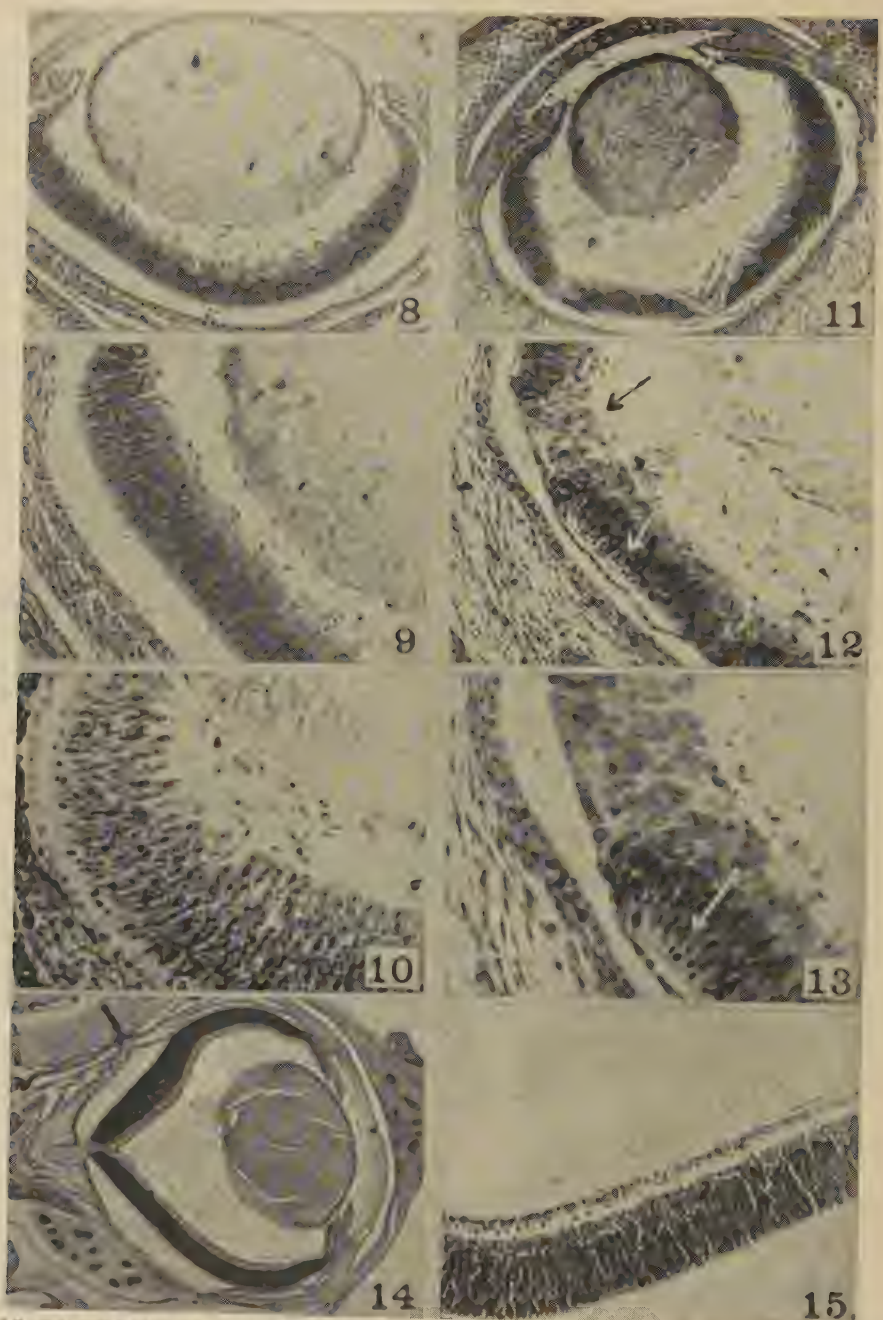

FIG. 8-13. Fetal eyes exposed at 13.5 days (Fig. 1, 2) to 300 r x-rays and fixed for study 72 hr later. Fig. 8-10 relatively less damaged, retina cleaned up by phagocytes and showing mitotic figures (Fig. 10) in the outer zone of nuclei destined to give rise to rods cells. Fig. 11-13 from similarly treated fetus, but eye shows persistent damage in form of areas of retina which are relatively devoid of cells. In addition there are adjacent areas (Fig. 12, 13) where the outer zone cells with mitotic figures are beginning the formation of rosettes.

FIG. 14, 15. Eyes at birth following x-irradiation to 300 r at 13.5 days, showing no gross residual damage. There is evidence of differentiation of retina to form inner ganglion cell layer and active mitoses in outer rods layer. None of the seriously radiation-damaged cells remain.

be only slightly affected. The lens, its epithelium and vascular tunic appear to be undamaged and intact, as is also the pigmented layer (Fig. 4).

If the fetal eye, exposed to 300 r at 13.5 days, is examined after 24 hours (Fig. 5-7) there continues to be considerable evidence of cellular damage although some of it has been removed by phagocytosis. The retina generally appears somewhat separated from the pigmented layer (Fig. 5) due, in all probability, to both a temporary cessation of growth and the loss of cells as well as to a temporary accumulation in this space of some sloughed off dead nuclei and cells. It seems evident (Fig. 5 and 6) that the outer nuclear zone has already shown some evidence of repair. This zone is quite free of dead cells and nuclei. The cells freed into the vitreous chamber are now gone but the inner nuclear zone still exhibits massive damage (Fig. 6). Phagocytes are active in ingesting the dead cells and nuclei (Fig. 7). Thus, by 24 hours there remains considerable evidence of radiation damage with initial repair evident in the presumptive rods zone.

By 72 hours following x-irradiation to 300 r of the fetus at 13.5 days the eye has all but regained its original activity and the presumptive retina is free of its dead cells and nuclei (Fig. 8-13). However, the range of variations in response within a single litter of mice identically treated is illustrated by comparing illustrations 8-10 with 11-13. All show the separation of the retina from the surrounding pigmented layer, and all show some retardation in development. But by this time phagocytosis has cleared away all the radiation-caused debris and the remaining living cells are free to progress toward differentiation. In Fig. 10 the outer nuclei of the presumptive rods layer are again in active mitosis without much evidence of lingering chromosomes or chromosome bridges. There is the earliest differentiation into inner and outer nuclear zones and there is little evidence of residual damage.

In litter mates and other fetuses similarly treated one occasionally finds an eye that has been unable to make the repair complete (Fig. 11-13) and, while the dead cells and cellular debris have been removed there are large portions of the retina which are not normal. Either they are relatively devoid of healthy cells (Fig. 12 black arrow) or the neuroblasts in active mitosis tend to form rosettes (Fig. 12, 13 white arrows). At such regions there is always a linearly-arranged group of cells which appear to have been detached from the outer margin of the retina. What these may become in the adult eye is under investigation. This situation appears to be the exception, because the majority of eyes examined at 72 hours post-irradiation have attained normal morphological contour and are only slightly retarded in development.

All eyes of such fetuses examined at birth (6-7 days after x-irradiation) appear to be quite normal (Fig. 14-15). There is a persistent separation of the retina from the pigmented layer but the retina itself is differentiated into a ganglion cell layer with its inner nerve fiber layer. The ganglion cell layer is distinctly separated from the nuclear layer of bipolar cells by the inner reticular layer. The nuclei of the rod cells have not yet been separated but mitosis is evident in this outer nuclear zone.

Discussion. The statement has often been made that the embryo and/or its cells are the most radiosensitive of all, and recently Hicks (1) has emphasized the extreme radiosensitivity of the neuroblast. The presumptive retina at 13.5 days fetal age consists almost entirely of neuroblasts, derived from primitive neurectoderm and destined to differentiate into retinal neurones. Since neurectoderm is relatively radioresistant those cells at 4 and 24 hours which do not exhibit damage may well be primitive neurectoderm. Those which are damaged by 4 hours post-radiation most likely are neuroblasts, certainly a long way from differentiated neurones. Since there is active phagocytosis at that time and still at 24 hours, there appear to be many dead cells and fragmented, pycnotic nuclei, one must conjecture that cells exposed in the neurectoderm stage immediately prior to their normal differentiation into neuroblasts similarly go to pieces at the time of such differentiation. Those cells which are still neuroectoderm and not immediately destined to differentiate into

neuroblasts are, no doubt, the majority of cells which remain to give rise to the regenerated retina. By 72 hours the developing retina again appears to be quite normal. By birth there is no gross evidence of radiation damage, even following the high level of exposure used.

That rosettes are formed is to be expected, Rugh(2), Hicks(1), Wilson *et al.*(3), in some instances. Wilson has described these as embryonic neoplasms or the origins of neoplasms, although they generally disappear by the time of birth. They are most certainly evidence of disorganization of outer zone nuclei which normally are integrated to form the rods of the adult eye. They always occur in the outer nuclear zone and are probably the result of a slowing down in proliferation of particular cells which then form the center of the rosette. This slowing down may well indicate damage to the chromatin material involved in mitosis of these cells.

It should be pointed out that this is *not evidence of "recovery"* in the sense that the damaged cells (or retina) regain their original integrity and activity. Certainly the most severely damaged cells have been discarded, and those which are able to survive minor damage remain to form the rosettes and relatively acellular regions of the retina. The change has been one of regeneration from the residual undamaged primitive neurectoderm cells, within the overall morphological plan of the developing eye. Apparently the radiation damage was essentially on the cellular level, since the general development of the eye remained quite normal.

It should also be emphasized that what holds true for the 13.5 day fetal eye may not hold true for the younger or older eye, as there would most certainly be a different ratio of neurectoderm, neuroblasts, and differentiating pre-neurones. A detailed study of other stages is being pursued and already indicates fundamental differences.

Had this study been based upon an analysis of the eyes at birth there would have been little or no evidence of damage to the eyes following 300 r at 13.5 days fetal age. Conversely, had the study been made only at 4 hours post-radiation, the conclusion might have been drawn that the eye was damaged beyond regeneration or repair. Since the study has been made in sequence from 4 to 72 hours and finally to 6 days (at birth) we have demonstrated that the embryonic eye is extremely radiosensitive, exhibiting mass cellular damage, but that it has remarkable powers of repair and reconstitution, resulting in what appears to be a normal eye at birth.

Summary and conclusions. 1) The 13.5 day fetus can sustain an exposure of 300 r and come to term. Its eyes, however, by 4 hours show massive damage to the presumptive retina such that approximately half of the cells are pycnotic or have fragmented nuclei. Many of these dead cells are sloughed off into the vitreous body or into the space between the retina and the pigmented layer. Both the inner and outer nuclear zones are involved, only the region of the ora serrata being rather free of damage. 2) At 24 hours there is evidence of continued necrosis, with about as many dead cells and nuclei as at 4 hours in spite of active phagocytosis. This suggests disintegration of some neurectoderm as it differentiates into neuroblasts. The outer neurectoderm of the presumptive retina appears to have begun proliferation. The vitreous body is rather free of dead cells and debris. 3) By 72 hours after exposure most fetal eyes have been able to repair the radiation damage and reconstitute the retina so that it appears quite normal. The eye as a whole is slightly smaller than that of the control, but the general morphology of the eye is normal. However, a few fetal eyes exhibit residual damage in the form of relatively cell-free areas and regions where the neuroblasts have been retarded in their proliferation and differentiation so as to cause the formation of rosettes. Since these were not seen at birth (6-7 days after exposure) it is presumed that they are temporary and that the retina adjusted to them. 4) Measured by the immediate (4 hr) effect, the fetal eye is very radiosensitive. However, the fetal eye exhibits remarkable reparative powers following severe radiation insult. This is achieved, not by recovery of radiation-damaged cells, but

by proliferation from the more radioresistant precursor neurectoderm cells.

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Influence of Adrenal on Decidual Development. (21774)

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It is known that decidual development can proceed normally in bilaterally adrenalectomized rats(1-4) and that the reaction can be decreased or inhibited in castrated, pseudopregnant animals on 1.5 mg progesterone daily by concomitant injections of 11-desoxycorticosterone-acetate, cortisone (cortone acetate) and adrenocorticotropic hormone (ACTH) (5). However, it also has been shown that a decidual reaction can be elicited in castrated, hypophysectomized rats receiving large doses of ACTH(6). The present paper reports an attempt to examine more closely the influence of the adrenal on decidual development.

Procedure. Estrous rats, 100 to 120 days old, were rendered pseudopregnant by electrical stimulation of the uterine cervix. All were castrated on the fifth day of pseudopregnancy and one horn of the uterus traumatized throughout its entire length by scratching the antimesometrial surface with a burred needle, while at the same time some of the animals also were bilaterally adrenalectomized. Immediately following these operations the animals were divided into groups, and while all were given progesterone some also received ACTH. Graded doses of the hormones were injected daily for 3 days and the animals killed on the seventy-second hour. The traumatized and normal horn of the uterus in each animal was weighed separately to the nearest tenth of a milligram.

Results. The tabular data presented indicates that daily doses of less than 1.0 mg of progesterone are more effective in promoting decidual growth when the adrenal glands are absent, while there is no significant difference in the response when larger amounts are given. It seems probable that these results may be due to a partial inhibition of the smaller doses of progesterone by adrenal cortical hormones and that the reaction is progressively reversed as the dosage of progesterone is increased. There also, of course, is the possibility that the larger doses of progesterone partially suppress adrenal function. If the adrenals are involved, it seems likely that their inhibitory influence should be increased by the administration of ACTH. This seems to be borne out by the fact that ACTH has a marked inhibitory action on decidual development when the adrenal glands are present, but is without effect in adrenalectomized animals. These observations seem to be in sharp disagreement with those of Lyons, *et al.*(6) who found that deciduomata could be induced in the uteri of castrated, hypophysectomized rats during ACTH treatment. However, this may be only apparent—as the experimental conditions of the 2 situations are quite different in several respects, the chief difference being that under our procedure a normal or moderately stimulated adrenal inhibited a given dosage of progesterone, while under their conditions it is more a problem of the physiology of a grossly exaggerated adrenal response to ACTH.

Summary. These data show that progesterone is more effective in the production of deciduomata in ovariectomized, adrenalecto-

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TABLE I. Influence of Progesterone and ACTH on Decidual Development in Ovariectomized and Ovariectomized-Adrenalectomized Rats.

Treatment	No. of rats	Uterine wt (mg) Control horn	Traumatized horn	% increase over control horn	Adrenal wt (mg)
mg					
Progesterone .25					
Adx.	10	104.7 ± 1.5*	307.2 ± 13.4	192.3 ± 15.4	
Control	8	100.0 ± 4	205.0 ± 8	115.0 ± 5	66.1 ± 1.9
Progesterone .50					
Adx.	7	107.0 ± 1.5	411.7 ± 16.6	285.0 ± 10.6	
Control	9	100.0 ± 3	290.0 ± 9	190.0 ± 6	64.0 ± 3.1
Progesterone .75					
Adx.	6	112.3 ± 2.7	460.0 ± 14.1	310.0 ± 16.4	
Control	13	107.0 ± 7	385.0 ± 12	260.0 ± 15	62.0 ± 1.6
Progesterone 1.0					
Adx.	8	104.4 ± 1.8	440.0 ± 7.8	323.0 ± 9.2	
Control	10	106.0 ± 5	437.0 ± 30	307.0 ± 13	56.6 ± 2.5
Progesterone 1.5					
Adx.	6	100.8 ± 5.2	471.1 ± 38.3	366.0 ± 19.7	
Control	37†	121.0 ± 7	546.0 ± 21	358.0 ± 35	59.1 ± 2.0
Progesterone 2.0					
Adx.	9	110.3 ± 1.1	490.1 ± 4.9	345.0 ± 10.1	
Control	12	120.0 ±11	520.0 ± 24	337.0 ± 32	54.0 ± 2.4
Progesterone .75					
ACTH .33					
Adx.	6	113.4 ± 3.2	443.5 ± 14.4	292.0 ± 19.4	
Control	6	113.4 ± 4.0	186.9 ± 9.5	65.3 ± 12.4	73.7 ± 1.2
Progesterone 1.5					
ACTH .33					
Adx.	8	110.9 ± 3.1	460.1 ± 8.4	327.0 ± 12.7	
Control	7	115.0 ± 1.9	282.0 ± 9.2	145.0 ± 7.5	75.7 ± 3.1
Progesterone 1.5					
ACTH .50					
Adx.	8	108.1 ± 1.1	476.1 ± 12.4	342.0 ± 13.5	
Control	8	113.2 ± 3.8	246.1 ± 16.7	117.7 ± 13.6	79.6 ± 2.0

* Avg ± stand. error.
† Results from several similar experiments other than those recorded here.
Adx. = Bilateral adrenalectomy. Control = Not adrenalectomized.
ACTH = Armour's Adrenomone—N11203.

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Reversal of the Actions of Tetraethyl Pyrophosphate in Surviving Mammalian Tissue. (21775)

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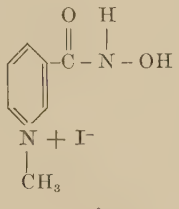
The development, chemistry, and pharmacology of the irreversible anticholinesterases have been extensively reviewed(1). More recent investigations(2) have been concerned with the nature of the chemical binding formed between these inhibitors and purified acetylcholinesterase. The results of these studies indicate 2 active centers on the enzyme surface to which substrate may be attracted. However, it appears that only one of these loci, the so-called esteratic site, is involved in the binding of inhibitors like the aryl and alkyl phosphates. Actually, the evidence points to the formation of a covalent linkage between a basic moiety of the enzyme and a phosphonium ion $[(RO)_2PO]^+$, the end result being a phosphoryl-enzyme complex. Utilizing these chemical theories, it was demonstrated *in vitro*(3) that suitably strong nucleophilic reagents, *e.g.* choline and hydroxylamine, could split the phosphoryl-acetylcholinesterase complex, resulting in enzyme reactivation. It was proposed that the "reactivator" attacked the phosphonium center, reorienting the attachment of the phosphonium ion from the esteratic site of the enzyme to the basic grouping of the reagent. A search for more effective "reactivators" resulted in the synthesis(4) of nicotinhydroxamic acid methiodide (NHMI) which has the following structure:



A quaternary ammonium compound was selected to enhance the possibility of reaction between reagent and phosphorylated enzyme. Using purified acetylcholinesterase from the eel, more than 90% of enzyme activity previously destroyed by either tetraethyl pyrophosphate (TEPP) or di-isopropylfluorophosphate (DFP), could be restored with NHMI.

The present experiments were made to determine if the results obtained with purified enzyme *in vitro* could be reproduced in isolated surviving mammalian tissue.

Methods. Young rabbits of either sex were killed by concussion and the heart and a strip of duodenum quickly removed. The heart was immediately immersed in normal saline and allowed to beat for 30 to 40 seconds subsequent to which the auricles were carefully excised from ventricular, fatty, and connective tissues. The isolated auricles were then bathed in an oxygenated 29.5°C modified Ringer-Locke solution containing in one liter: 7.9 g NaCl, 0.42 g KCl, 0.24 g $CaCl_2$, 0.20 g $MgCl_2$, 2.1 g $NaHCO_3$, and 1.8 g glucose. The lumen and exterior of a 3 to 5 cm strip of duodenum was washed thoroughly with normal saline and suspended with lumen opened in the muscle chamber. The strip was bathed in an oxygenated 37.5°C. Krebs-Ringer solution which was prepared by adding 1.3% $NaHCO_3$ (192 ml) and glucose (1.8 g) to one liter of the stock solution (0.90% NaCl, 100 parts; 1.15% KCl, 4 parts; 1.22% $CaCl_2$, 3 parts; 2.11% KH_2PO_4, 1 part; 3.82% $MgSO_4 \cdot 7H_2O$, 1 part). Both the intestinal and auricular mediums were maintained at pH 7.4. Each organ was attached to a counterweighted isotonic lever for kymograph recordings. *TEPP*† was prepared fresh each day in normal saline from a stock solution containing 5% TEPP in propylene glycol. NHMI‡ was dissolved in distilled water and neutralized with 0.5 *M* NaOH so that a final concentration of 75 to 100 mg/ml was ob-

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‡ Obtained through the courtesy of Dr. I. B. Wilson, Columbia University, College of Physicians and Surgeons.

TABLE I. Effect of NHMI on Auricular Slowing Produced by TEPP, ACh, and MCh.

Drug and dose	No. of trials	Min. after NHMI admin.	No. of reversals	Avg % slowing Before NHMI	Avg % slowing After NHMI
TEPP—.2 μg/ml	20	5	20	69.0	40.5
	20	30	20	69.0	31.8
	10	180	10	77.9	22.6
ACh—.2 to 1 μg/ml	6	5	0	9.6	33.1
	6	30	0	9.6	27.5
	6	60	0	9.6	35.6
MCh—.16 to .8 μg/ml	9	5	1	30.0	33.5
	9	30	1	30.0	26.7
	8	60	1	29.1	28.1

tained. In all experiments the dose of NHMI used was 3 mg/ml of organ bath, a dose which proved to be optimal. Drugs were prepared so that the volumes added to the bath never exceeded one ml. *Test organs* were allowed to stabilize in the muscle chamber for at least one hour before initiation of the experimental period. For the evaluation of drug effects rate of auricular contraction and height of intestinal tone were measured, both these factors becoming constant during the stabilization period. In all cases the effect of NHMI was studied first and later its effect determined on the induced cholinergic activity in the same tissue; in this way each tissue piece served as its own control. In addition, 6 controls for each drug used were run on separate test organs to study character and duration of action. Often, several trials were made on the same organ; between each trial the organ was thoroughly washed and a 30 to 60 minute period allowed for restabilization.

Results. A. Effect of NHMI on TEPP-treated auricles. In every case the TEPP-induced diminution of auricular rate was significantly reversed by NHMI. In Table I this effect is quantitated by comparing the per cent slowing before and after administration of NHMI. It may be seen in this table that the reversal of TEPP inhibition was already marked at the end of 5 minutes following administration of the compound, the negative chronotropic effect of TEPP being opposed by nearly 30%. Furthermore, the auricular rate continued to speed until at the end of a 3-hour period the average TEPP-induced inhibition was antagonized by more than 50%. In one trial, in which contraction was completely halted by TEPP, only 40% slowing remained at the end of 5 minutes following addition of the reagent. *Contraction amplitude,* decreased by TEPP, was also restored by NHMI. Control amplitude was often regained 5 minutes after adding the compound and was always attained at the end of 30 minutes. The recovery followed a progressive course, and in most cases amplitude increased above the control level by the end of the observation period. *Negative inotropic* and chronotropic actions of TEPP were completely maintained over periods of 6 to 8 hours in control experiments. Thus, the pronounced reversal of these effects by NHMI is made to appear even more striking. In the previously untreated auricle, NHMI sometimes (7 out of 25 trials) resulted in a transient increase in rate subsequent to an initial depression. In none of these instances was the increase of sufficient magnitude to suggest a direct action of the compound as responsible for the reversal phenomenon. That this accelerator response to NHMI was a fortuitous affair was substantiated by the fact that a second test on the same organ usually failed to reproduce the effect.

B. Effect of NHMI on acetylcholine and methacholine-treated auricles. There has accumulated an abundance of evidence to support the hypothesis that the effects of TEPP, at least in such isolated organs as used in these studies, are mediated entirely by acetylcholine (ACh). On this basis, it might be thought that the reversal phenomenon observed with NHMI is attributable to some anticholinergic action of the latter. To test this idea experiments were made on the effect

TABLE II. Effect of NHMI on Increased Intestinal Tone Produced by TEPP, ACh, and MCh.

Drug and dose	No. of trials	Min. after NHMI admin.	No. of reversals
TEPP .02 to .2 μg/ml	23	60	16
ACh .4 μg/ml	8	60	0
MCh .01 to .28 μg/ml	6	30	0

of NHMI upon functional changes produced by ACh and Methacholine (MCh). It is significant that in no instance did the compound antagonize the ACh-induced inhibition of auricular rate; in fact, it may be seen from Table I that NHMI enhanced ACh action. The absence of an anti-cholinergic property is indicated again in the MCh studies; in all but one of 9 trials NHMI neither potentiated nor reversed the negative chronotropic response to MCh.

C. Effect of NHMI on TEPP-treated intestine. In Table II it can be seen that NHMI reversed the effects of TEPP on intestinal strips in about 70% of the trials. Thus, the increased tone gradually declined, and simultaneously the amplitude of the rhythmic contractions reappeared or increased. In 50% of the cases in which NHMI was effective the tone regained control level. In 4 of the negative trials there also occurred a decrease of tone after addition of the compound, albeit a relatively minor one. In *control experiments* all actions of TEPP—increased tone, periodic spasms, reduction or complete cessation of rhythmic contraction—were completely sustained over the 3-hour test periods. Furthermore, the direct action of NHMI upon the intestinal strips was insignificant; only a minor and transient increase in tone and amplitude was observed, the latter more marked than the former.

D. Effect of NHMI on ACh and MCh-treated intestine. The results with intestinal strips (Table II), like the findings with the isolated auricles, did not indicate any anti-cholinergic effect of NHMI.

Discussion. The results show clearly that NHMI can reverse the effects of TEPP to a significant degree on the isolated surviving mammalian tissue used in this investigation. This is consistent with the *in vitro* demonstration of the reactivation of TEPP-inhibited acetylcholinesterase. Accordingly, it was shown that reversal was not the result of a parasympatholytic effect of NHMI; the insignificance of an extraneous action of NHMI was also indicated. However, the experiments described herein do not directly confirm the chemical mechanisms by which this compound is postulated to reactivate the inhibited enzyme.

During these experiments, the cholinergic properties of NHMI appeared either as a direct transient effect or were evidenced through an enhancement of ACh action. The presence of the quaternary ammonium structure in NHMI undoubtedly explains this cholinergic property, but the extent of this action is exceedingly weak when compared with more common cholinergic agents like ACh and MCh. However, despite this minimal cholinergic action, summation with TEPP was never apparent, and reversal of the TEPP-induced effects always predominated. The chief contribution of the quaternary ammonium center probably is to increase the affinity of NHMI for the phosphorylated enzyme.

Considering the minute quantities of TEPP used in these studies, one may well inquire as to the reason for the comparatively huge amount of NHMI needed for reversal. However, the dose of 3 mg/ml of organ bath is not very large when compared relatively with the amounts needed for enzyme reactivation in the *in vitro* studies with pure acetylcholinesterase. It is possible that excessive destruction or hydrolysis of the compound may occur(5), hydroxylamine being one of the end products.

Summary and conclusions. 1. Experiments were made to determine the effect of a new compound, nicotinhydroxamic acid methiodide (NHMI), on isolated mammalian tissue treated with TEPP. 2. NHMI possesses the property of reversing to a significant degree the TEPP-induced actions in isolated auricles and intestinal strips of the rabbit. 3. That this effect of NHMI was not the result of an anti-

cholinergic action was shown by the inability of the compound to reverse the effects of ACh or MCh. 4. That this reversal phenomenon was not the result of a direct action upon the tissue was shown by the negligible response of the organs to the compound alone. 5. These studies indicate that reversal of TEPP-induced effects by NHMI may be due to a true chemical reactivation of cholinesterase.

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The Mechanism of Satiety: Effect of Glucagon on Gastric Hunger Contractions in Man.* (21776)

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(Introduced by Harold G. Wolff.)

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Bulatao and Carlson reported that intravenous injection of glucose can abolish gastric hunger contractions in fasting dogs(1). Because of the difficulty in reproducing these findings, and the "unphysiologic" nature of the injection procedure, subsequent attempts to relate satiety to blood glucose levels were not successful(2-5).

Recent availability of a highly purified preparation of glucagon (hyperglycemic glycogenolytic factor)(6,7) has made possible a new approach to this problem. Accordingly, a study has been made of the effect of glucagon administration on gastric hunger contractions and the experience of hunger in human subjects.

Procedure. Seven healthy adult volunteers served as subjects, 3 of them on 2 separate occasions. Each subject was in the postabsorptive state at the time of study. At the beginning of the experiment a Levine tube with attached balloon was inserted into the stomach. The balloon was inflated to a pressure of 10 cm of water, and the tube withdrawn until resistance was encountered at the cardia. The tube was then taped into place at the nose. Gastric contractions were recorded on a kymograph by means of a water manometer. An infusion of isotonic sodium chloride solution was begun and base-line

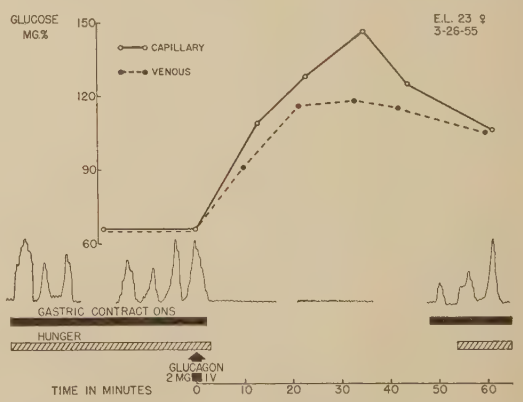

FIG. 1. (Subject E. L.). Inhibiting effect of 2 mg of glucagon on gastric hunger contractions and experience of hunger. As capillary-venous differences decrease, gastric hunger contractions return even though glucose levels are well above fasting values. The sample tracings are from a kymograph revolving at a speed of one inch per min. Black bar represents actual time relations of the contractions to glucose levels and experience of hunger.

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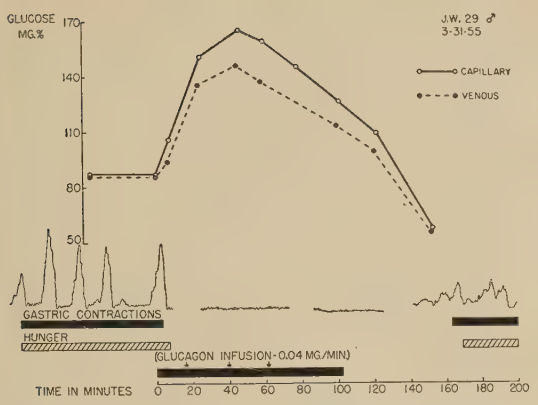

FIG. 2. (Subject J. W.). Effect of a constant infusion of saline containing 4 mg of glucagon on gastric hunger contractions and experience of hunger. Appreciable capillary-venous glucose differences are maintained during the infusion period. Gastric hunger contractions return only after discontinuance of glucagon infusion.

capillary and venous blood samples were drawn. Control periods lasted up to 4 hours, during which the subject's pattern of gastric activity was ascertained, as well as the effect on gastric motility of a rapid injection of 10 ml saline containing 2 ml glucagon diluting fluid through the infusion tubing. Glucagon was administered during vigorous gastric contractions according to 3 different schedules: 1. On 7 occasions in 6 subjects, 2 mg glucagon in 10 ml saline was injected intravenously through the infusion tubing over a one-minute period. Venous and capillary blood samples were drawn every 10 minutes for 30 minutes and then at 15- to 25-minute intervals (Fig. 1). 2. Two experiments involved repeated intravenous injections of smaller amounts of glucagon. One subject received 0.5 mg of glucagon on 3 occasions, while another subject was given 5 injections of 0.125 mg glucagon. In both subjects, capillary and venous blood samples were obtained at frequent intervals. 3. One subject (J. W.), received 4.0 mg glucagon intravenously in 290 ml saline by constant (Bowman pump) infusion over a 102-minute period. Capillary and venous samples were drawn at intervals indicated in Fig. 2. Venous blood was drawn without stasis from an antecubital vein, and capillary blood (which is identical in glucose content with arterial blood(8)) was obtained almost simultaneously by direct pipetting from a finger tip of the same extremity after cutaneous puncture. Glucose in whole blood was determined by the method of Somogyi(9,10) as modified by Nelson(11). All determinations were done in duplicate on 0.2 ml samples. The glucagon‡ was a highly purified preparation made by the method of Staub *et al.*(12), and contained 0.005-0.05 unit of insulin per mg glucagon.

Results. On all 16 occasions when glucagon was administered intravenously, abolition of gastric hunger contractions occurred within 3 minutes after the start of glucagon injection. The sensation of hunger disappeared or diminished shortly thereafter. Control injections of saline and glucagon diluting fluid, as well as repeated venipunctures and finger punctures had no demonstrable effect on either phenomenon.

Following injections of a single 2-mg dose of glucagon, both capillary and venous glucose levels increased rapidly, reached a peak at approximately 30 minutes after injection, and then began to fall. Maximum increases in capillary glucose over control values averaged 77.0 $\pm$ 9.2 mg %.§ At the same time the average capillary-venous glucose difference increased from a control value of 2.4 $\pm$ 3.2 mg % to 18.0 $\pm$ 6.9 mg %. On the falling limb of the curve capillary-venous glucose differences decreased (Fig. 1), and in 6 of the 7 experiments there was a return of gastric hunger contractions and the experience of hunger during the period of observation. In each of these cases the capillary glucose value was still well above the control level (mean 63.0 $\pm$ 9.1 mg % above fasting), but capillary-venous glucose differences measured when hunger contractions returned averaged 6.0 $\pm$ 1.4 mg %.

This same pattern of gastric response and hyperglycemia was observed in the two subjects given multiple intravenous injections of small amounts of glucagon. It is worthy of

‡ Glucagon was supplied through the courtesy of Dr. W. R. Kirtley, Eli Lilly and Co.

§ Stand. dev. $= \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}}$.

note that the degree of hyperglycemia decreased with each successive injection, as did the length of time during which gastric contractions were abolished.

Gastric contractions were inhibited during the entire 102 minutes of the continuous infusion of glucagon in subject J. W. (Fig. 2). During the first 40 minutes of infusion, capillary and venous glucose levels were rising, and for the last 60 minutes they were falling. Throughout the period of infusion the capillary-venous glucose difference was not less than 11 mg per 100 ml. After discontinuance of the glucagon infusion there was acceleration in rate of fall of glucose levels and a decrease in the capillary-venous glucose differences to below 6 mg %. These changes were accompanied by a return of gastric hunger contractions.

Discussion. Inhibition of gastric hunger contractions invariably followed glucagon administration in this series. Such reproducibility is in contrast to the inconstant effect of glucose injection on gastric motility(1-5), and raises the question of how glucagon can induce such a consistent response. Four possibilities deserve consideration: 1. the influence of the absolute level of blood glucose, 2. the effect of a glucose gradient, 3. a direct effect of glucagon, and 4. the degree of glucose utilization.

Since the hyperglycemia following glucagon injection was substantially less than that following intravenous injections of glucose which failed to abolish hunger contractions(13), it seems probable that the level of the blood glucose *per se* is not the determining factor. This is consistent with Scott's findings that the presence of hunger does not correlate with venous glucose levels(5). The same criticism applies to the view that the glucose gradient is critical since gradients obtained by intravenous injection of glucose are much steeper than those following glucagon administration(13).

Glucagon may abolish hunger contractions by a mechanism which is independent of its effect on carbohydrate metabolism. The available data do not permit a decision on this matter.

It has long been suspected that the degree of glucose utilization might determine satiety (1). However, lack of a satisfactory method for determining glucose utilization has hampered investigation of this problem. Increased accuracy of methods for the measurement of blood glucose(9-11) has made feasible the use of peripheral capillary-venous glucose differences as a qualitative index of glucose utilization. This index has proved useful in correlating carbohydrate metabolism with states of hunger and satiety(14). Moreover, administration of glucose appears to abolish hunger contractions only when the hyperglycemia is accompanied by increased capillary-venous glucose differences(15).

Recently, it has been demonstrated that glucagon tends to promote peripheral utilization of glucose as measured by peripheral capillary-venous differences(16,17) in addition to its better-known capacity to raise blood glucose levels by accelerating hepatic glycogenolysis.‖ In each of the experiments reported herein, comparatively large capillary-venous glucose differences were present during periods of gastric quiescence. Accordingly, the inference seems justified that the consistent gastric inhibition induced by glucagon may be related to an effect on peripheral glucose utilization.

These findings are compatible with the "glucostatic" theory of regulation of food intake(18,19). This theory postulates that hunger is mediated by hypothalamic "glucoreceptors" responsive to depletion of carbohydrate reserves, while satiety results from the increased utilization of glucose associated with replenishment of such stores.

Previous successful attempts to abolish hunger contractions by means of intravenous glucose administration have been dismissed as not pertinent to the normal satiety mechanism because of the use of strongly hypertonic solutions, a peripheral site of administration, and

‖ The only other physiologic agent capable of raising blood glucose levels by hepatic glycogenolysis is epinephrine. Unfortunately, it has not been possible to interpret the effect of epinephrine on hunger because of the numerous and varied physiologic responses to its administration.

the possibility that the glucose so injected differs from that normally present in blood. The findings reported in this paper obviate such objections since the pattern of hyperglycemia induced by glucagon is similar to that occurring under natural conditions following ingestion of food. Indeed, such a pattern may be a component of the normal satiety mechanism.

Summary. Administration of glucagon intravenously to 7 normal subjects resulted in prompt abolition of gastric hunger contractions on each of 16 occasions. This was associated with decrease in the experience of hunger and coincided with a rise in blood glucose levels and an increased peripheral capillary-venous glucose difference. Fall in blood glucose levels with decreasing capillary-venous differences was associated with a return of hunger contractions and intensification of the experience of hunger in 14 of the 16 occasions. Such a pattern may be a component of the normal satiety mechanism.

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Mucopolysaccharides in Tissue Cultures of Human and Mammalian Synovial Membrane.* (21777)

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The following studies are based on 546 tissue cultures of the synovial membrane from human, monkey, rabbit, ox, horse, pig, chicken and rat joints. The controls consisted of 82 cultures from the periarticular connective tissues.

Procedure. The *basic medium* contained fowl plasma, 30%; chicken embryo extract (EE 100), 2.5%; and Tyrode solution 67.5%. The supernatant fluid consisted of homologous or heterologous serum 50%, Tyrode solution 47.5% and chick embryo extract 2.5%. The concentration of embryonic extract was kept low in order to suppress the common type of fibroblasts and to minimize

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the introduction of mucopolysaccharides contained in the chick extract. Tissue fragments were washed repeatedly in Tyrode solution for from one-half to one hour to remove adherent synovial fluid. The supernatant fluid in the Carrel flasks was changed once weekly, in the roller tubes twice weekly. The period of culturing varied between 5 and 15 weeks. Only a few characteristic features of the growth of synovial lining are outlined here. In contradistinction to the round form of the explant of periarticular connective tissues, the explants of the synovial lining assumed usually an irregular form due to small villi protruding from the periphery. Migration of cells occurred from the tip as well as from the side of the villi, thus increasing very markedly the surface of new growth, which consisted of a mixture of spindle-shaped fibroblasts and pleomorphic cells similar to those observed by Vaubel(1) and Murray *et al.*(2). The polygonal cells frequently formed pavement-like sheets. They contained numerous cytoplasmic coarse granules which stained rapidly with neutral red and metachromatically with toluidine blue in high dilutions. The spindle-shaped fibroblasts showed a few fine granules which took up the neutral red slower and exhibited faint or no metachromasia with toluidine blue.

Determination of mucopolysaccharides in the supernates. Addition of acetic acid to supernates which contained mucopolysaccharides produced a ropy precipitate which did not dissolve in excess acetic acid, but did so readily in KOH. This test requires from ¼ to ½ ml of fluid. A modification described by Kling(3) can be carried out with only a drop of fluid, delivered into a test tube containing 2% acetic acid. Mucopolysaccharides in the presence of protein cause the formation of a definite opaque sac or tube. Fluids containing large amounts of hyaluronic acid form a sac which adheres to the surface of the acetic acid. As the quantity of hyaluronic acid decreases the sac wall becomes thinner and the sac tends to drop to the bottom of the tube. The precipitation tests were positive in 162 (66.7%) out of 216 supernates withdrawn from synovial tissue cultures. In 54 (33.3%) they were either negative or showed a homogenous turbidity. The supernates from the controls were negative. Supernates which gave positive precipitation tests were depolymerized by testicular and bacterial hyaluronidase. Quantitative determination of hyaluronic acid in the supernatants by a modification of the turbidimetric method described by Schmith and Faber(4) yielded from 4 to 15 mg per 100 ml. Relative viscosities were determined by a modification of the Mann(5) capillary viscometer. The mean relative viscosities of 27 supernates which gave negative acetic acid precipitation tests was 1.15 ± .05, while 48 fluids with positive tests had a significantly higher mean viscosity of 1.56 ±

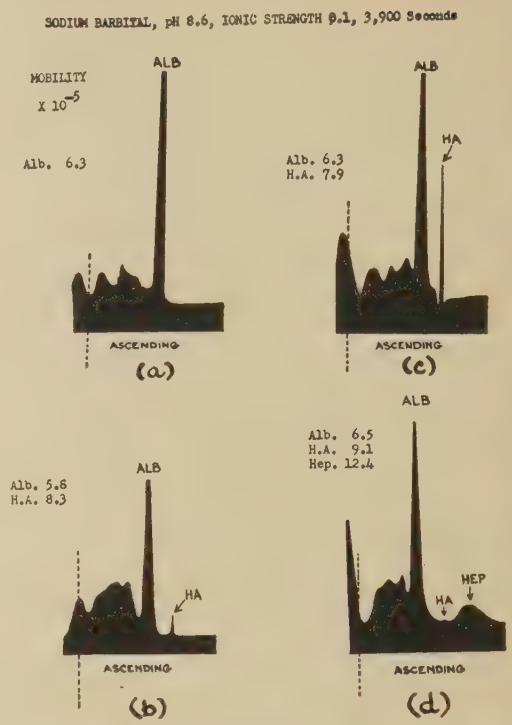

FIG. 1. Electrophoretic patterns. (a) Supernatant fluid negative for mucoproteins. ALBumin highest peak with the fastest mobility; boundaries with slower mobility are globulins. (b) Supernatant fluid positive for mucopolysaccharides, hyaluronic acid (HA) fastest peak in front of ALBumin. (c) Supernatant negative for mucopolysaccharides with 0.4% hyaluronic acid added. Note high peak with the mobility in range of HA in (b). (d) Supernatant positive for mucopolysaccharides in all patterns. HEParin 0.4% was added. Note the HEP boundary in front of HA. Note the absence of a chondroitin sulfuric acid peak usually found between the peaks for HA and HEP.

0.26. Metachromasia was tested with toluidine blue. None of the supernatant fluids from synovial tissue cultures and control developed metachromasia. Heparin (as little as 5 μg per ml) and other sulfonated mucopolysaccharides showed strong metachromasia with toluidine blue.

Electrophoretic Studies. To further establish the nature of the mucopolysaccharides found in the supernatants, electrophoretic patterns were obtained with a Perkin-Elmer Tiselius apparatus.

Supernates with negative acetic acid precipitation tests gave protein patterns with peaks characteristic of the globulins and albumin. The latter gave the highest and fastest moving peak (Fig. 1a). Supernates which gave a positive precipitation test showed a new peak in front of the albumin (Fig. 1b). The mobility of this component was in the range of hyaluronic acid verified by the addition of purified hyaluronic acid to a negative supernatant fluid (Fig. 1c). None of the supernates exhibited a peak with a faster mobility than that for hyaluronic acid. The sulfonated mucopolysaccharides such as chondroitin sulfuric acid or heparin have a faster mobility than the hyaluronic acid. This is seen in the electrophoretic pattern obtained when heparin is added to a tissue culture supernate positive for hyaluronic acid (Fig. 1d).

Summary. 1. Growth of human and animal synovial membrane in tissue cultures showed characteristic differences from control cultures of periarticular tissues. 2. Presence of hyaluronic acid in about two-thirds of supernates of tissue cultures from synovium was demonstrated by precipitation reactions with acetic acid, enzymatic reactions, viscosity determination, tests for metachromasia and electrophoretic patterns. 3. Quantitative determinations by a turbidimetric method yielded a concentration of hyaluronic acid from 4 to 15 mg %. 4. Tests were negative for sulfonated mucopolysaccharides such as chondroitin sulfuric acid or heparin. 5. Control cultures of periarticular connective tissue were negative for hyaluronic acid and sulfonated mucopolysaccharides.

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Simple Method for Enhancing Development of Acute Disseminated Encephalomyelitis in Mice.* (21778)

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The production of acute disseminated encephalomyelitis (ADE) in mice has hitherto depended on multiple injections of encephalitogenic materials such as normal mouse brain or its proteolipide A + B fraction. The number of injections was arbitrarily set at 6, at weekly intervals, although some injected animals showed signs after fewer doses. The period of observation in those receiving the full injection course was consequently at least 40 days. During the past 2 years a study was undertaken on simplification of this procedure, with the aim of reducing the number of injections and the time of observation. This study has made it possible to devise a more economical method of producing ADE, based on the "adjuvant" action of *H. pertussis* vaccine(1,2) combined with the use of 2 intracutaneous inoculations of the encephalitogenic material in a Freund-type, water-in-oil emulsion, adjuvant

* The technical assistance of Sophie L. Skadorwa is gratefully acknowledged.

(3). Both the vaccine and the intracutaneous route have been reported previously as enhancing the response to an antigenic stimulus(1-5). Since the basis of the mechanism for development of ADE in mice was postulated as being due to hypersensitivity(6,7), it was thought that both devices might be applied to increase the incidence of reactors and to shorten the duration of the experiments.

Materials and methods. Previous reference has been made to the difference in susceptibility of 2 strains of mice. The BSVS strain is susceptible and the BRVR is resistant(6,8). The production of ADE in susceptible mice was hitherto achieved by 6 or less subcutaneous injections at weekly intervals and the materials injected were mouse brain, or its proteolipide A + B fraction first described by Folch and Lees(9), combined with a Freund-type adjuvant. The mixture of proteolipide and that adjuvant will be designated hereafter as PA. The methods, dosage, and other general procedures, including description of the experimental disease, have already been reported in detail(6,10). In the present study PA, prepared as previously described, was injected intracutaneously into several sites on the animals' back. The hair was removed first from 4- to 8-week-old mice by an Oster A-2 electric clipper. With a one-inch, 21-gauge needle attached to a tuberculin syringe, 0.3 ml was delivered as a total dosage for each course of injections divided into 3 or more sites (blebs). This amount contained 4.2 mg proteolipide A + B. Those animals which received *H. pertussis* vaccine (Phase 1, 60,000 million/ml, Lederle) were injected intraperitoneally with 0.1 ml at varying periods before PA was given, or intracutaneously, when admixed with the latter, as indicated in Tables I, II, and III. The following injection procedures were examined: 1) The encephalitogenic property of a single intracutaneous injection of PA, with and without prior exposure to *H. pertussis* given intraperitoneally. 2) The response to PA when introduced at varying time intervals of 0, 1, 2, and 4 days after *H. pertussis*. 3) The frequency and time of development of ADE produced by 2 inoculations of PA. 4) The influence of *H. pertussis* vaccine on the activity of PA given 4 days later with a second injection 3, 5, 7, 10, and 13 days after the first. All animals were observed for manifest signs of ADE. Thirty days was arbitrarily chosen as the time limit for an experiment, although in the present series several tests were completed within 21 days. At the end of this period, if an animal had shown no clear neurological signs, its brain was removed and examined for histopathological lesions. Positive signs were also confirmed by characteristic pathological findings. Accompanying each experiment in which animals received PA intracutaneously was a control group of 7-10 mice which were injected with the same material subcutaneously up to 6 times at weekly intervals. Since all controls were 100% positive, they are omitted from the tables.

TABLE I. Effect of *H. pertussis* Vaccine on Production of ADE in BSVS Mice Receiving 1 or 2 Injections Intracutaneously of Proteolipide A + B at 7 to 13 Days Interval.

No. of injections	Without vaccine, No. pos./No. inj.	With vaccine,* No. pos./No. inj.
1	2/31	7/11
2	35/40	20/20

* *H. pertussis* given intraperitoneally 4 days before 1st injection. For control tests in all tables see text.

Results. The experiments carried out in this study could be divided into 3 general groups.

The first test (Table I) was designed to examine the preliminary effect of *H. pertussis* vaccine on the production of ADE in BSVS mice receiving later 1 or 2 injections intracutaneously of PA. When 2 inoculations of the latter mixture were given, the interval between the treatments was 7 to 13 days. It will be noted that without the use of the vaccine and after a single dose of PA alone, 2 of 31 inoculated mice showed ADE; whereas after 2 exposures to PA 35 of 40 reacted. But with *H. pertussis* vaccine given intraperitoneally 4 days before a single inoculation of PA the incidence of ADE was 7 of 11 inoculated mice; after 2 doses of PA all of 20 treated animals showed the disease.

The second experiment on the effect of the time interval between the intraperitoneal injection of *H. pertussis* vaccine and a single in-

TABLE II. Effect of Time Interval between Intraperitoneal Injection of *H. pertussis* Vaccine and a Single Intracutaneous Injection of Proteolipide A + B.

Exp. No.	Vaccine inj. (days before)	No. pos./No. inj.
39	0*	7/12
36	1	5/7
37	2	6/7
28	4	7/11

* *H. pertussis* vaccine was mixed with proteolipides A + B—adjuvant mixture and injected intracutaneously together. In experiments 36, 37, and 28, the vaccine was given before the proteolipide.

tracutaneous injection of PA is summarized in Table II. The enhancing effect of the *H. pertussis* vaccine was noted even when added to PA and both were injected intracutaneously. Also, the effect of the vaccine was evident when the vaccine was given at an interval up to 4 days before the PA. In all the experiments more than 50% of the mice inoculated responded to a single dose of PA.

The results of the third series of tests are given in Table III and exhibit the effect of the time interval between the first and second intracutaneous injection of PA in BSVS mice, with or without preliminary *H. pertussis* vaccine. They indicated that a 3-, 5-, 7-, 10-, or 13-day interval was equally effective in the fairly uniform production of ADE in *pertussis* injected animals. Only one failed to show ADE within 30 days when the interval between the first and second exposure to PA was 5 days; otherwise the incidence was 100%.

To recapitulate the results, the intracutaneous route of inoculation was more effective than the subcutaneous for development of ADE in mice. Without use of *H. pertussis* vaccine, a single intracutaneous inoculation of PA induced ADE in 6% of the mice; with the preliminary exposure to the vaccine and with a subsequent dose of PA, the incidence of reactors rose to 60%. Without vaccine and with 2 spaced injections of PA the incidence was 90%; with vaccine 100%. When all mice reacted as in the last instance, the experiment was completed at 21 days instead of 40-50 days for the earlier method of repeated subcutaneous injections.

TABLE III. Effect of Time Interval between First and Second Intracutaneous Injection of Proteolipide A + B in BSVS Mice, with or without Preliminary *H. pertussis* Vaccine.

Interval between 1st and 2nd inj. (days)	Without vaccine, No. pos./No. inj.	With vaccine,* No. pos./No. inj.
3	3/ 9	5/5
5		6/7
7	6/ 8	7/7
10	6/ 7	6/6
13	23/25	7/7

* *H. pertussis* given intraperitoneally 4 days before 1st injection.

Discussion. It has already been postulated that ADE in mice could result from development of hypersensitivity(6,7)—a hypothesis offered as a basis for the mechanism of similar reactions in other experimental animals(11). It is therefore interesting that in the present studies enhancement of ADE could be brought about by the preliminary use of *H. pertussis* vaccine and by the intracutaneous way of inoculation, both being factors which promote hypersensitivity.

Conclusion. By manipulations here described it was possible to induce acute disseminated encephalomyelitis in mice in a shorter time than heretofore, with fewer inoculations and with uniform response. The improved method consists of the intraperitoneal injection of *H. pertussis* vaccine followed by 2 spaced intracutaneous injections of proteolipide A + B prepared in a Freund-type adjuvant.

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Antiaccelerator (Anticonvertin) Activity of Canine Plasma and Serum.* (21779)

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Many workers have emphasized the importance of the dynamic balance of accelerators and inhibitors in the process of blood coagulation. This paper describes studies of an antiaccelerator activity, first reported 2 years ago(1), which appears to participate in this dynamic balance. The antiaccelerator effect was observed accidentally in some experiments in which mixtures of citrated canine hemophilic plasma, cephalin and a serum accelerator (SA) preparation rich in convertin were incubated and recalcified at intervals. The clotting time became longer as the incubation time increased. This was found to be the result of progressive inactivation of SA by the plasma. Normal canine plasma was found to cause similar inactivation of SA. Further studies showed that albumin fractions prepared by salting-out procedures contained all of the antiaccelerator activity.

Materials and methods. Serum accelerator (SA) was prepared as follows: native normal dog plasma was clotted with 1/20 volume of beef lung thromboplastin; after 20 min at 37°C, the serum was oxalated and adsorbed for 30 min at 28°C with 20 mg $BaSO_4$ per ml; the $BaSO_4$ was washed thoroughly with 0.02 M sodium oxalate and eluted with 0.11 M sodium citrate (10 ml per g $BaSO_4$); after dialysis (Nojax casing, Visking Corp.) against normal saline for 16 hrs at 4°C, the eluate was stored at –20°C. It was rich in convertin (40 SPCA units per ml)(2). The results of tests for other clotting factors in the SA preparation were as follows: (a) Prothrombin: less than one unit per ml by the modified 2-stage method(3). (b) Thrombin: equal volumes of SA and fibrinogen did not clot in 30 min at 28°C. (c) Thromboplastin: SA had no measurable effect in 30 min on prothrombin conversion in platelet-poor native hemophilic plasma(4). (d) AcG(5): none detected. *Normal serum,* obtained from platelet-rich canine plasma kept for 2 hrs at 37°C after clotting, was mixed with ¼ volume of 0.1 M sodium oxalate. *Antiaccelerator fractions* were prepared from oxalated canine plasma or serum after adsorption with $BaSO_4$ (100 mg per ml). The fractions were precipitated between 50 and 80% saturation with neutral saturated ammonium sulfate, dissolved and dialyzed for 4 to 16 hrs at 4°C against normal saline. *Globulin fractions* were prepared in similar manner from precipitates obtained by 50% saturation of plasma with $(NH_4)_2SO_4$. *Fibrinogen*(6) and *Factor V*(6) were made from oxalated plasma obtained from a dicoumarolized hemophilic dog after adsorption with 100 mg $BaSO_4$ per ml. This type of plasma was used to minimize contamination with other clotting factors. *Cephalin* was prepared from dog brain (7). *Prothrombin* was prepared from normal dog plasma(8). *Buffered calcium* reagent was obtained by mixing 70 ml 0.11 M $CaCl_2$, 60 ml 0.25 M imidazole buffer at pH 7.3 and 50 ml normal saline. *Citrated hemophilic dog plasma* was obtained as described earlier(3). *Thrombin*: Topical Thrombin (Parke Davis) was dialyzed against distilled water, clarified by repeated centrifugations at 35,000 *g* for 30

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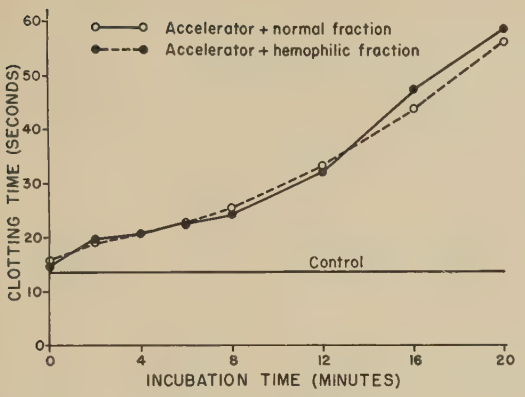

FIG. 1. Comparison of antiaccelerator activity of normal and hemophilic plasma fractions. The following mixtures were prepared and incubated at 28°C: a. 0.6 ml SA + 0.4 ml *normal* albumin fraction in saline, equivalent to 0.03 ml plasma; b. 0.6 ml SA + 0.4 ml *hemophilic* albumin fraction, equivalent to 0.03 ml plasma; c. 0.6 ml SA + 0.4 ml saline *(control)*. At intervals, 0.1 ml incubation mixture was added to 0.15 ml of a reagent mixture consisting of 0.2 ml 300 unit per ml prothrombin, 0.4 ml Factor V, 1.6 ml fibrinogen, 0.2 ml 0.3% cephalin and 2.6 ml oxalated saline (0.005 M sodium oxalate in saline). After addition of 0.1 ml 0.02 M $CaCl_2$, clotting time was obtained.

min, and diluted with normal saline to a concentration of 100 NIH units per ml. *PTC* was prepared from normal dog plasma(9). *Antithrombin* was measured by a modified method for antithrombin III(10).

Results. Demonstration of antiaccelerator activity in fractions from normal and hemophilic plasma and normal serum. Antiaccelerator activity was studied in a two-stage system: (1) SA was progressively inactivated by incubation with an antiaccelerator preparation; (2) at intervals, the residual SA activity was detected by its effect on the clotting time of a system of semipurified reagents, consisting of prothrombin, Factor V, cephalin, fibrinogen and $CaCl_2$. The rate of increase in clotting times after incubation furnishes a measure of the antiaccelerator activity of the preparation. This is shown in Fig. 1, where the antiaccelerator activity of normal and hemophilic plasma albumin fractions is demonstrated. It is observed that these fractions caused progressive loss of SA activity at approximately equal rates. In other experiments, normal serum albumin fractions were found to possess an antiaccelerator activity similar to that of the plasma fractions shown in Fig. 1. Normal and hemophilic plasma globulin fractions, on the other hand, were inactive.

Effect of antiaccelerator on thromboplastin and cephalin. Fig. 2 illustrates the lack of effect of a normal plasma antiaccelerator fraction on the thromboplastic activity of a beef lung extract. A similar lack of effect was observed when the antiaccelerator fraction was incubated with cephalin (0.02%); in the measurement of SA activity in these experiments, cephalin was omitted from the reagent mixture (Fig. 1). Clotting times were in the range of 115 to 130 sec.

Rate of SA-antiaccelerator reaction. Fig. 3 shows the rapid loss of activity which occurred when SA was incubated with an antiaccelerator preparation. The residual SA in the incubation mixtures was measured by its effect on the clotting time of a mixture of hemophilic plasma, cephalin and $CaCl_2$. In this experiment, one-half of the initial activity had disappeared in 2 min. The control, SA incubated with saline, showed no loss of activity.

Assay of antiaccelerator activity. On the basis of studies of the type shown in Fig. 3, an assay of antiaccelerator activity was devised. By serial dilution of plasma or an active plasma fraction, that amount of antiac-

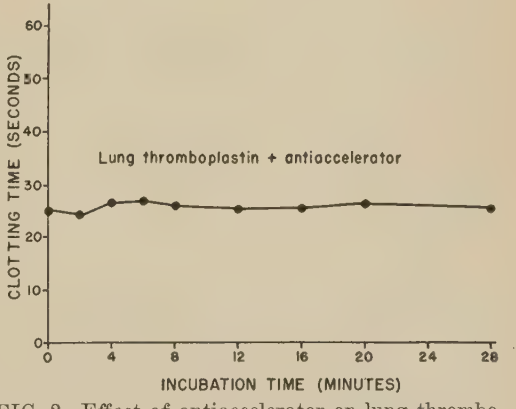

FIG. 2. Effect of antiaccelerator on lung thromboplastin. A mixture of 0.1 ml saline, 0.2 ml antiaccelerator fraction, and 0.7 ml of a 1 to 32 saline dilution of beef lung thromboplastin(6) was incubated at 28°C. At intervals, 0.1 ml of the mixture was added to the reagent mixture described in Fig. 1.

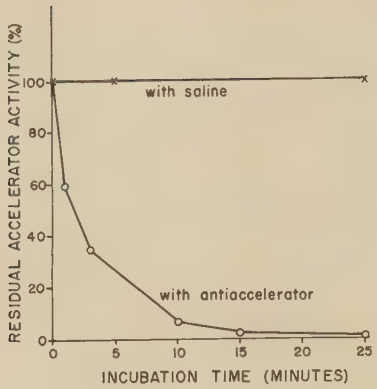

FIG. 3. Rate of inactivation of SA. The following mixtures were prepared and incubated at 28°C: a. 0.9 ml SA + 0.1 ml normal albumin fraction, equivalent to 0.02 ml plasma; b. 0.9 ml SA + 0.1 ml saline. At intervals, 0.1 ml of incubating mixture was transferred into a tube containing 0.1 ml citrated hemophilic plasma and 0.1 ml 0.015% cephalin. Clotting time was obtained after addition of 0.1 ml buffered $CaCl_2$. Per cent residual SA was estimated from a calibration curve obtained by testing serial dilutions of SA in the above clotting system; undiluted SA gave a clotting time of 7.5 sec. (equivalent to no destruction of SA), no SA gave 150 sec. (equivalent to complete inactivation of SA).

celerator is found which will cause 50% inactivation of a standard accelerator preparation in 30 min. This degree of antiaccelerator activity is contained in normal plasma diluted over 100-fold. In Table I is shown the use of this assay procedure in the determination of the relative antiaccelerator activity of normal and hemophilic plasma. It will be noted that the plasma concentration in the incubation mixtures which caused 50% inactivation of SA is between 0.25 and 0.5%. By interpolation, this value is found to be 0.33% for each plasma. Again, as in Fig. 1, the antiaccelerator activity of the two types of plasma was approximately the same.

TABLE I. Assay of Normal and Hemophilic Plasmas for Antiaccelerator Activity.

Components of incubation mixture*			
Plasma			Clotting time,† sec.
Type	Conc., %	SA, ml	
Normal	.5	.05	46
"	.25	.05	39
"	.125	.05	35
Hemophilic	.5	.05	49
"	.25	.05	37
"	.125	.05	34
None (control ≅ 100% SA)		.05	29
" (" ≅ 50% ")		.025	42
" (" ≅ 0% ")		None	153

* Incubation mixtures made to constant volume, 0.1 ml, by adding imidazole buffer. Incubation time, 30 min., 28°C in siliconed tubes. Diluted with 0.9 ml saline to slow reaction before testing.

† Clotting times determined by adding 0.1 ml diluted incubation mixtures to 0.1 ml 0.015% cephalin, 0.1 ml hemophilic citrated plasma and 0.1 ml buffered $CaCl_2$.

Antiaccelerator and antithrombin activities: Properties of the same or different entities? In a series of experiments simultaneous antithrombin and antiaccelerator assays were done on the same fractions. Over 90% of both plasma antithrombin and antiaccelerator were found in the fraction precipitated between 60 and 80% saturation with $(NH_4)_2SO_4$. Further fractionation showed over 50% of both activities in the fraction separated between 70 and 80% saturation. Both activities were retained after dialysis for 3 days. Further attempts were made to separate these two activities without success. Like antithrombic activity, antiaccelerator activity is almost completely destroyed by heating at 65°C for 12 min. Plasma extracted twice with diethyl ether at room temperature lost more than 90% of both activities. The $(NH_4)_2SO_4$ precipitate obtained between the ranges of 70 to 80% saturation was successively extracted with 78, 75, 72, and 70% saturated $(NH_4)_2SO_4$. A similar precipitate was extracted five times with 72% saturated $(NH_4)_2SO_4$. The extracts tested after dialysis showed no separation of antithrombin and antiaccelerator. In view of these similarities, it might be suggested that a trace of thrombin in the SA preparation was the sole factor which was inactivated. However, when SA and thrombin with equal effectiveness in the SA test systems were each tested with fibrinogen, the thrombin gave a clotting time of about 30 sec, while the SA gave no clot in 30 min. Furthermore, addition of thrombin to the incubated SA-antiaccelerator mixtures prior to testing (thrombin concentration of 0.1 unit per ml) failed to cause reactivation. These experiments indicate that the SA activity is not due to thrombin. Thus the antiaccelerator activity must represent inactivation of a nonthrombic accelerator, probably convertin.

Antiaccelerator influence on thrombin tide.

TABLE II. Decrease in Thrombin Tide following Incubation of SA with Antiaccelerator Preparation.

Incubation,* min.	Thrombin (units/ml) formed† in 2 min.	5 min.	10 min.	20 min.
0	18	25	20	12
25	4.0	6.4	7.2	5.2

* Incubation mixture contained 0.2 ml SA preparation, 0.2 ml antiaccelerator preparation, and 0.6 ml 0.9% NaCl. Mixtures were incubated for 0 or 25 min. at 28°C in siliconed tubes.

† At end of incubation period, 1.5 ml solution was added which contained 0.9 ml 300 unit/ml prothrombin, 0.1 ml 0.3% cephalin, and 0.5 ml Factor V. The mixture was then recalcified with 1.0 ml of 0.02 M $CaCl_2$. The recalcified mixture was incubated at 28°C and at 2, 5, 10, 20 min., 0.35 ml aliquots were pipetted into siliconed tubes containing 0.1 ml 0.11 M sodium citrate. Thrombin units were determined immediately.

Table II illustrates a series of three-stage experiments in which the effect of antiaccelerator on thrombin tides was studied. When SA was inactivated by incubation with antiaccelerator preparation in the first stage, both the speed of thrombin formation and thrombin yield were greatly reduced. In these experiments, the amount of antiaccelerator preparation and antithrombic activity are kept constant. With the SA preparation free of detectable thrombin, the findings in Table II reemphasize that the antiaccelerator is not acting simply as a neutralizer of thrombin. Rather they suggest that antiaccelerator functions by reducing SA effect on prothrombin conversion, thus limiting the magnitude of the thrombin tide.

Discussion. It is suggested that the inactivation of the SA preparations described here results from loss of convertin activity. The term SA is retained, however, as loss of activity of one or more other factors may be concerned. One possibility is PTC; however, substitution of a PTC preparation for SA in the test system failed to cause accelerated clotting, even in the absence of antiaccelerator. Antiaccelerator activity(1) may have been anticipated by Owren(11) as well as others, who observed a slow disappearance of convertin-type of activity of serum.

The similarity of antiaccelerator to plasma antithrombin suggests that these two activities may be functions of a single protein entity, particularly since recent evidence suggests that convertin may be a non-thrombic derivative of prothrombin(12). Other inhibitors in plasma—antitrypsin, antifibrinolysin, and heparin cofactor—have also been reported to be in the albumin fraction, and have not been clearly separated from each other. The recent work of Lyttleton(13) indicates that antithrombin and heparin cofactor, although albumins as judged by salting-out procedures, are alpha globulins. An interesting aspect of our experiments is that antiaccelerator, unlike antithrombin, is readily demonstrable at very low concentrations of plasma (Table I).

The rate of inactivation of SA (Fig. 3) points to a rapid turnover of this factor during clotting. Since the factor is known to persist in serum, there must be an SA tide comparable to the thrombin tide described by Smith(14), with only a trace of the potential SA present in an active form at any time. Antiaccelerator may have an important physiologic role in maintaining the fluidity of the blood. Prompt removal of the SA activity would help protect prothrombin from rapid conversion to thrombin. Such an effect may explain, at least in part, the relatively slow conversion of prothrombin in plasma compared to purified systems(15).

Summary. 1. Canine plasma and serum possess potent antiaccelerator activity, probably anticonvertin. This activity is retained after dialysis, but is thermolabile and is inactivated by multiple ether extractions. It is found in the precipitate obtained between 50 and 80% saturation with ammonium sulfate. Separation from antithrombin has not been accomplished. 2. An assay procedure for determining relative antiaccelerator activity is described. 3. Normal and hemophilic plasma and normal serum all have approximately the same antiaccelerator activity. 4. It is suggested that this new activity limits the tide of SA during clotting, analogous to antithrombin and the thrombin tide.

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Effects of Salts and Colloids on Potency of Botulinum Toxin.* (21780)

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Halter(1), Neter(2) and also Traub, Hollander and Friedemann(3) have reported that lower values were obtained by mouse assays of tetanus and botulinum toxins in saline than in solutions of meat broth, peptones or serum. Halter and Neter indicated that the titrations in saline were too low, but the others presume that a potentiation afforded by the broth and serum was responsible for the higher assays. Coleman(4) found that leukocytes, mixed with the filtrates of cultures of *Clostridium botulinum,* increased the measure of their toxicity and that intramuscular injections of peptone subsequent to the subcutaneous injection of the toxin into guinea pigs hastened the death of the animals. Jensen(5) reported that normal serum and heterologous antisera enhanced the evident toxicity of botulinum toxin, tested in white mice. He administered the serum "adjuvant" before or after the injection of the toxin, or he injected a mixture of the toxin and serum. Wentzel, Sterne and Polson(6) found a high toxicity when the dilutions of Type D toxin were made with gelatin-phosphate solutions. They stated that this diluent appeared to have a potentiating effect on the toxin similar to that reported by Traub, *et al.*(3). Littauer(7) "consistently" obtained about 10% higher values with a solution of gelatin and potassium acid phosphate than with acetate buffer as the diluting medium. Before the mice were injected, the dilutions of the toxin at pH 6.8 stood for one hour at room temperature. The latter investigator pointed out that more constant values were obtained when the diluent was kept at 8°C during titrations than at higher temperatures. His assays indicated 40-50% lower activity at 25°C than at 8°C and complete inactivation at 80°C.

Materials and methods. The toxin used in these studies was prepared by cultivation of *Clostridium botulinum,* Type A in the manner described by Lowenthal and Lamanna(8). The toxin was precipitated by acid, the method of concentration used by Sommer(9), and a stock suspension was prepared by suspending a measured amount of this precipitate in distilled water. One batch of the stock suspension of the toxin was used to test the effect of the various diluents discussed later in this report. It assayed approximately 6 x 10^5 mouse units per ml when the dilutions were made as described below in the standard autoclaved gelatin-phosphate buffer. Sterile

* We wish to express our gratitude to Anthony C. Tancredi, Lorenzo J. Gault and Joseph J. Chambliss for their skillful technical assistance.

TABLE I. Effect of Various Colloidal Diluents on the Assay of Botulinum Toxin in Mice.

Diluent pH 6.9			
Colloid	g/100 ml phosphate buffer	Sterilized	Relative potency*
Sheep serum	Whole	Seitz filter	123.1
" " albumin	3.	*Idem*	109.0
Human " "	3.	"	122.4
Gastric mucin	5.	Autoclave	322.5‡
Idem	4.	*Idem*	194.9‡
"	2.	"	177.5‡
"	.2	"	140.8
Gelatin	4.	Seitz filter	162.0
Idem	4.	Autoclave	122.0
"	.2	Seitz filter	158.4‡
"	.2	Autoclave (standard diluent)	100.0
"	.2	Autoclave, inj. followed by inj. of phosphate buffer†	17.4‡

* Relative or comparative potencies associated with the use of the various diluents are expressed in terms of percentage of the potency obtained by use of the standard diluent, 0.2% gelatin in 1.0% phosphate buffer.

† The potency was reduced 82.6% when the injection of the toxin in "standard diluent" was followed by intraperitoneal injections of 0.5 ml of 0.5% phosphate buffer 3, 6, 20, 26 and 48 hr later.

‡ These values were found by statistical analysis to vary significantly from the value of 100 for the standard diluent.

technic was used throughout the course of these experiments. The assays were made by intraperitoneal injections of 0.5 ml doses of serial dilutions in 16 g white mice† and the LD_{50} at 4 days was recorded as the measure of the potency. Most of the mice that died succumbed within 24 hours. Unless pH adjustments were made, the suspended toxin did not dissolve completely in water in the usual time allowed, and erratic and unpredictable assays resulted. When the stock suspension was diluted first with 0.2% gelatin in 1.0% phosphate buffer at pH 6.9 - 7.0, called the "standard diluent" for convenience, solution was more rapid but inconsistencies in the assays were still observed. The question arose in the course of these experiments as to whether reduction of the concentration of the gelatin by one or two 10-fold dilutions in water was thorough enough to afford true comparison of the effect of the final diluents. To overcome these difficulties the 1.0 ml portion of the stock suspension was added to 9.0 ml phosphate buffer, pH 7.0 and neutrality was maintained until it was entirely dissolved. The toxin dissolved rapidly, and immediately after complete solution it was diluted 100-fold with water. The dilutions in the various diluents adjusted to pH 6.9 were prepared from this water solution for assay in mice.

Results. As indicated in Table I, the assay of the toxin was significantly higher when the standard diluent was sterilized by Seitz filtration than by autoclaving at 15 lb pressure for 15 minutes. The titer was not altered significantly when the standard autoclaved gelatin-phosphate diluent was replaced by sheep serum or sheep albumin, sterilized by Seitz filtration, or by 0.2% gastric mucin in phosphate buffer, sterilized by autoclaving. Peptone, casein, beef broth and other similar substances gave approximately the same results but are not shown in Table I. Replacements of these substances by gastric mucin of 2, 4 or 5% concentration in 1.0% phosphate buffer solution resulted in a significantly higher titer of the toxin. When the test mice were injected with a water solution of the toxin with or without added gelatin or other protein, the titer did not vary significantly from that obtained when the standard diluent, 0.2% gelatin in 1.0% phosphate buffer was used. These results are shown in Table II. However, the assay of the toxin was significantly lower when the dilutions were made with the sodium chloride solutions or the acetate, phosphate or citrate buffers alone

† Webster strain mice were used. For convenience serial dilutions were made at about 25°C.

TABLE II. Comparative Assays in Mice of Botulinum Toxin in Various Diluents, with and without Gelatin.

Diluent, pH 6.9 autoclaved	Conc., g/100 ml H_2O	Relative potency* Diluent with no gelatin	Relative potency* Diluent with 0.2% gelatin added
Distilled water	—	95.9	109.6
Sodium chloride	.85	8.6	83.1
" "	5.0	14.5	79.4
Sodium citrate—citric acid buffer	1.	6.3	95.5
Idem	3.	46.7	100.0
Sodium acetate—acetic acid buffer	1.	14.1	95.5
Idem	5.	35.9	102.2
Sodium acid phosphate buffer (stand. diluent)	1.	21.4	100.0

* Potencies expressed in terms of comparative values based on the results obtained with the "standard diluent," 0.2% gelatin in 1.0% phosphate buffer assigned the value 100. The potencies measured by use of the diluents listed, except water, were found by statistical analysis to be significantly lower when the gelatin was omitted.

than when gelatin was added to these diluents.

Discussion. The variation in the results obtained when the original dilution of the acid stock suspension was made in H_2O without further pH adjustment appears to have been due to inadequate solubility at the low pH.

Whether the gelatin solution enhances the toxicity of the botulinum toxin or whether the buffers or saline lower the potency is still a matter of conjecture. The latter explanation is suggested by the results shown in Table I where it is seen that several injections of rather large doses of phosphate buffer in the mice after administration of the toxin in standard diluent (gelatin-phosphate) lowered the fatality rate about 80%. On the other hand, it is possible that the correct explanation is, as Sterne(6) suggested, that a potentiating effect of the gelatin-phosphate diluent might be due to a disaggregation of the toxin into smaller units much like dissociation of proteins under the influence of other proteins, amino acids and simple inorganic salts. Such dissociation of proteins has been discussed by Svedberg and Pederson(10) as well as by a number of other workers. Wagman and Bateman(11) have shown that under certain conditions of pH and ionic strength botulinum toxin exists in the form of two distinguishable polyphase components. The higher LD_{50} obtained when the higher concentrations of gastric mucin were used in the diluent very likely may be due to an effect on the physiology of the mouse rather than on the toxin. The possibility is suggested that the mucin, gelatin, serum proteins, etc., rendered the animal more susceptible to the action of the toxin. Mucin has been shown to lower the resistance of animals to infection(12). The evident potencies of the botulinum toxins estimated by assays in mice certainly are dependent upon the constituents of the solutions used to dilute the toxins injected into the test animals. Which results should be chosen as the true potency is still a matter of interpretation. It is concluded that a standardized diluent such as an autoclaved solution of 0.2% gelatin in 1.0% phosphate buffer, must be chosen to obtain comparable results. The adoption of a standard procedure for the assay of botulinum toxin is suggested.

Summary. Attention is called to discrepancies arising in mouse assays of botulinum toxin when the method of dilution and choice of diluent are not standardized. As much as 50-fold variation in the toxicity determinations resulted from the use of various diluents in the preparation of the serial dilution. Explanations of some of the discrepancies are suggested.

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Treatment of Dried Whey with Various Solvents; Effects upon the Growth of Chicks.* (21781)

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Studies on natural feedstuffs by Hill(1) showed that dried whey contained a factor which promoted the growth of chicks fed rations containing certain samples of soybean oil meal. Additional evidence for the presence of a chick or poult growth-promoting factor in dried whey was presented by Reed *et al.*(2), McGinnis *et al.*(3), Scott(4), Fuller *et al.*(5), Fischer *et al.*(6), and Combs *et al.* (7). In one report of work on unidentified factors, dried whey did not produce a growth response (Sunde *et al.*(8)).

Attempts were made to study the value of whey as a source of unidentified factors by chick assay using a ration containing a purified protein source and other ingredients of synthetic origin. Early studies on fractionating whey indicated that treatment with various solvents increased the growth-promoting value of dried whey and anhydrous polar solvents were less effective than solvents containing water. With this information, further studies were made to confirm the role of water in improving the growth-promoting value of dried whey for chicks.

Procedures. Methanol-extracted soybean oil meal was used as a protein source in experiments 1 and 2 and isolated soybean protein (Drackett C-1) at an equivalent protein level was used in the diets for Exp. 3, 4, and 5. When Drackett protein was used the basal ration was as follows: Drackett protein (C-1), 22 g; cellu flour, 5 g; soybean oil, 3.5 g; calcium carbonate, 2.5 g; mineral mixture(9), 2.5 g; vitamin mixture(9), 2 g; dicalcium phosphate, 2 g; choline chloride (25%), 0.8 g; DL-methionine, 0.5 g; glycine, 0.3 g; vit. A carrier (10,000 units/g) 0.1 g; vit. D_3 carrier (1,500 units/g) 0.1 g; d-alpha tocopheryl acetate carrier (44 units/g) 0.1 g; biotin, 0.02 mg; vit. B_{12}, 1 μg; and corn starch (pearl) to total 100 g. In Exp. 4 and 5 the basal ration contained 10 mg of aureomycin per 100 g. Day-old chicks were divided into comparable groups of 10 birds each and were fed the rations for periods of 24, 21, 24, 28, and 25 days in the successive experiments. In Exp. 4 and 5 duplicate groups of 10 birds each were used. They were housed in heated batteries with raised wire floors and were provided feed and water *ad libitum*.

Solvents as indicated in Table I were added to samples of dried whey (commercial preparation) in the ratio of 2 liters of solvent to 375 g of whey. The mixtures were heated for one hour at 50-55°C and filtered by suction when separation of the extract and residue was made. The preparations were mixed with the other portions of the diet and were dried overnight in an oven at 50°C. Dried whey or preparations from it were added to the basal ration at 7.5% of the ration or equivalent.

Results. In the feeding tests with chicks (Table I) dried whey gave only a slight or no growth response. When the whey was extracted with 70% methanol, 70% ethanol, or 70% isopropanol, the extract was more active than the original whey. The extract and residue together were also more active than the original whey while the residue alone was, in most cases, only slightly better than the original whey. Treatment with water in an amount equivalent to that of the alcohol-water mixture was effective also in improving growth above that observed with the original whey. Water added to the basal ration caused no increase in growth in Exp. 3 but caused a slight increase in Exp. 4 and 5.

Discussion. The reason for the improvement in the chick growth-promoting activity of whey by treatment with water is not

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TABLE I. Effect of Treatment of Dried Whey with Various Solvents upon Growth of Chicks.

Supplement	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
			Avg wt, g		
0	217	158	225, 234	213	194
Dried whey, untreated	230	151	234	234	209
70% methanol extract		181			
" residue		167			
" extract + residue	250	174			
70% ethanol extract		171			
" residue		179			
" extract + residue		182	272		
70% isopropanol extract		177			
" residue		161			
" extract + residue		176			
Water treated whey			274	256	224
Water (added to basal)			233	224	200
Duration of experiment, days	24	21	24	28	25

known. The original whey solids were in the presence of water before the material was dried. It thus seems that alteration in some of the constituents of the whey solids by the drying process and restoration to its original form by water treatment is the best hypothesis to offer at the present time. Investigations on the mechanism by which water treatment improves the nutritive value of whey are being continued.

Summary. Dried whey treated with 70% methanol, 70% ethanol, 70% isopropanol or an equivalent amount of water improved the growth of chicks to which it was fed more than an equivalent amount of untreated dried whey.

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Action of Parenteral Trypsin on Experimentally-Induced Edemas of Different Types in Rats. (21782)

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It has been reported(1,2) that trypsin inhibits the formation of local egg-white edema in rats. These findings have been confirmed by Adamkiewicz *et al.*(3) for local edema produced by egg-white, kaolin and yeast filtrate in normal and adrenalectomized rats. Trypsin as well has been reported to inhibit the generalized edema produced by dextran(4). On the other hand Hardy *et al.*(5) observed no effect of a trypsin-in-oil preparation on local edema induced by dextran in adrenalectomized rats. Kleinfeld and Habif(6), while reporting an inhibitory effect of trypsin on clinical inflammation, found no action with small doses on animals with the Selye granuloma pouch. The present work was under-

TABLE I. Effect of Trypsin on Edema Produced by Various Phlogistic Agents.

Agent	Dose trypsin (mg/kg)	Administration	Wt diff. (saline leg-edema leg) (g) Control	Experimental	Inhibition (%)
Yeast	5	S.C.	1.35 ± .10	1.00 ± .10	25
Dextran	5*	"	1.26 ± .14	1.14 ± .17	0
	20*	"	1.26 ± .14	.98 ± .14	25
	5	"	1.26 ± .18	1.26 ± .05	0
	20	"	1.26 ± .18	.76 ± .17	40
	5*	I.P.	1.20 ± .10	1.34 ± .23	0
	20*	"	1.20 ± .10	.88 ± .15	20
	5	"	1.20 ± .10	.80 ± .12	25
	20	"	1.26 ± .14	.66 ± .21	45

* Trypsin-in-oil preparation (Parenzyme) was used.

taken to study the actions of trypsin on several different types of experimentally-induced edema, and to attempt to reconcile the discrepancies in published reports on the action of trypsin in laboratory animals.

Methods. Measurements of extent of local edema were carried out by weighing the amputated foot, as previously reported(2). Rats weighing 90-100 g were employed; all figures are averages for groups of 5 animals. Local edema was produced by injection of the agents used into the foot as follows: Commercial dextran, 0.125 ml of a 3% solution average M.W. 75,000(5) and Brewer's yeast, 0.4 ml of a 20% suspension. In each case the animals were sacrificed and the legs amputated 1.5 hours after administration of the edema-producing substance. Unless otherwise stated, the trypsin was injected in one dose ½ hour before injection of the phlogistic agent. Generalized edema was produced by intraperitoneal injection of 1 ml of egg-white or dextran 200 mg/kg, as a 6% solution in saline. Trypsin was administered ½ hour before the egg-white or dextran. Extent of edema was determined at intervals by gross observation. An all-or-none measurement was used to overcome the inaccuracies inherent in the gross observation technic; animals with any perceptible swelling of the extremities were considered to be positive. Figures therefore represent the number of positive responses in each experimental group.

Results. Table I gives the results obtained with 2 different phlogistic agents on local administration. Obviously the edemas produced by these agents are not equivalent as far as their inhibition by trypsin is concerned. Egg-white edema is inhibited by trypsin(2) at a concentration of 2 mg/kg; the dextran edema does not respond to trypsin in either water or oil on subcutaneous administration at a level of 5 mg/kg. The edema produced by yeast is apparently quite sensitive. Adamkiewicz *et al.* have reported its inhibition by a total dose of a trypsin-in-oil preparation amounting to 0.81 mg/kg.

The increase in effect obtained with the aqueous trypsin on intraperitoneal administration suggested that speed of absorption might be a factor affecting the results; this is undoubtedly of prime importance in an acute experiment. A number of animals were treated with trypsin at varying intervals before the production of a dextran edema, in order to determine whether the half-hour interval routinely used allowed enough time for

TABLE II. Effect of Time of Administration on Action of Trypsin on Local Dextran Edema.

Time (hr) of administration (before edema production)	Dose (mg/kg)	Wt diff. (saline leg-dextran leg) (mg) Control	Experimental	Inhibition (%)
.5	5*	1.26±.14	1.14±.17	0
.5	20*	1.26±.14	.98±.14	25
.5	5	1.26±.18	1.26±.05	0
.5	20	1.26±.18	.76±.17	40
3	5*	1.14±.05	.96±.15	15
3	20*	1.14±.05	.88±.06	25
3	5	1.14±.05	.82±.06	30
3	20	1.14±.05	.62±.18	45
24	5*	1.46±.17	1.18±.12	20
24	20*	1.26±.05	.67±.13	45
24	5	1.20±.10	.90±.20	25
24	20	1.26±.05	.52±.08	60

* Trypsin-in-oil preparation (Parenzyme) was used.

TABLE III. Inhibition of Generalized Edema by Trypsin.

Phlogistic agent	Dose trypsin (mg/kg)	Inhibition of edema (%)
Egg-white	10	0
	20	25
	40	50
	50	75
Dextran	5	0
	10	0
	20	50
	50	100

the development of an optimal blood level. This was felt to be of particular interest with the trypsin-in-oil preparation, where some delay in absorption might reasonably be expected to occur. The results from this experiment are presented in Table II. All doses of trypsin were given subcutaneously. The figures show that time of administration is definitely a factor in the results. Both preparations used, which showed no effect at the 5 mg level when given ½ hour before edema development, give a definite effect when a 3-hour interval is allowed to elapse. The effect of the 20 mg doses is at a high level after 24 hours; 5 mg doses show only a slight effect at this time interval and presumably the peak effect has been passed somewhere between 3 and 24 hours. The results in general lead to the conclusion that the assumption that a system is saturated with trypsin at an arbitrarily chosen dosage schedule may be unjustified in a given experimental technic.

In Table III are presented results obtained with generalized edemas produced by intraperitoneal injection of egg-white and dextran. In all cases the trypsin was injected subcutaneously, and the results are from observations made 3 hours after injection of the phlogistic agent, at which time edema was invariably well-marked in the extremities of all controls. It is apparent that a higher dose of trypsin is required to inhibit an edema of this type than is necessary for a purely local edema. This once again points out the fact that experimentally produced edemas of different types may react differently quantitatively to trypsin. The results obtained with the generalized dextran edema confirm work done by Cohen *et al.*(4). These workers noticed no difference in the response of normal, adrenalectomized or hypophysectomized rats in this phenomenon. Adamkiewicz *et al.*(3) also found no difference in response to trypsin of adrenalectomized rats with local egg-white edema.

The findings as a whole indicate that trypsin is effective in inhibiting several different types of experimentally-produced edema. They also show that the different types of edema are not necessarily equivalent quantitatively in their response to trypsin, and that therefore dosages and dosage schedules which have been found to inhibit one type of edema will not necessarily prove effective against another. Thus the results obtained by Hardy *et al.*(5) may be explained on the basis of insufficient dosage. This obviously applies with at least equal force to clinical edemas as opposed to those produced in the laboratory.

Summary. Trypsin has been shown to inhibit the development of several types of experimental edema. Quantitative responses differ according to the phlogistic agent being used and its mode of administration.

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Clot Density Determination of Fibrinogen in Experimental Myocardial Infarction. (21783)

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Previous studies disclosed that the fibrinogen concentration reflects the severity of acute myocardial infarction(1,2). In a group of 50 patients the mortality rate was 42.1% when the maximum fibrinogen concentration exceeded 800 mg % whereas only 9.7% of the remaining patients died. In coronary insufficiency no elevation of the fibrinogen concentration was encountered. These observations suggested that the fibrinogen concentration may reflect the extent of a myocardial infarct. A technic of rapid fibrinogen determination (3) was utilized for clinical studies(1,4,5). If applicable to dog plasma, the size of experimentally produced myocardial infarction could be correlated with the fibrinogen level by this procedure, called the clot density method.

Methods. a) 50 specimens of citrated dog plasma were diluted with saline to 80%, 60%, 40% and 20%. Clot densities were determined in duplicate for each fibrinogen concentration. b) Clot densities of 50 samples of citrated human plasma were studied in similar fashion. Clot density differences of 2 successive dilutions were plotted against the clot density representing 100% fibrinogen concentration.

Material. 1. Twenty dogs weighing from 15 to 20 kg each, were subjected to ligation of the descending branch of the left coronary artery just below the bifurcation of the main artery. 2. In 20 dogs a suture was placed just below the origin of the septal artery from the descending branch of the left coronary artery. 3. In 10 dogs, a sham-operation was performed, including pericardiotomy. The coronary arteries, however, were not violated. After i. v. administration of sodium pentobarbital in a dosage of 30 mg per kg, an endotracheal tube was inserted and attached to a mechanical respirator. The left side of the chest was explored through the 4th intercostal space, thereby permitting exposure of the left auricle and left coronary artery. Ligatures were placed at the prescribed sites around both, artery and vein or else, following isolation, the artery alone(6). Clot density determinations of fibrinogen were carried out on all dogs prior to operation and every day postoperatively. All animals were sacrificed within 8 to 15 days and their hearts carefully examined as to the occlusion of the coronary artery, the extent of necrosis and the phase of healing of the infarction.

Results. The *in vitro* studies of the clot density of human as well as canine plasma revealed that the decrement appeared rather constant. The difference between the clot densities of 100% and 80% fibrinogen concentration was the same as between those of 40% and 20% fibrinogen in either species. Superimposition of scattergrams correlating 100% and 20% fibrinogen concentration revealed an identical distribution of the values for canine as well as human plasma along a straight line.

The mean maximum fibrinogen concentration in animals with high ligation was 588 mg %, in those with low ligation only 516 mg % as compared to 408 mg % in animals with a sham-operation (Fig. 1).

Gross inspection of hearts from animals 5 days after high coronary ligation disclosed wedge-shaped extensive replacement of the anterior wall by a gray-white moderately firm, vascular tissue extending from the ligature to the apex. A similar type of tissue was seen after low coronary ligation, but involving a smaller area. In dogs sacrificed between the 12th and 15th day, the heart muscle was replaced by a pearly-gray, firm, demarcated scar.

Microscopic studies in all 40 dog hearts with coronary ligation revealed either granulation tissue when the animals were sacrificed

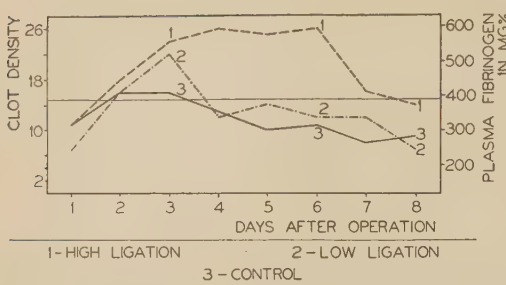

FIG. 1. Mean fibrinogen levels of 20 dogs with high coronary ligation, 20 dogs with low ligation and 10 control animals.

between the 8th and 12th post-operative days, or extensive fibrosis in those sacrificed at a later date. No myocardial changes were noted in the control animals.

Discussion. The linear relationship between fibrinogen concentration and clot density of either canine as well as human plasma and the identical distribution of values correlating 100% with 20% fibrinogen concentration along a straight line sufficiently warranted the application of the same formula for canine as for human plasma, namely $F = 18d + 120$, wherein F indicates the fibrinogen concentration in mg % and d the difference in optical densities before and after clotting, or "clot density"(3).

The 3 surgical procedures leading to various degrees of cellular necrosis also produced different fibrinogen levels. The sham-operation caused sufficient tissue damage to stimulate fibrinogen production(7), whereas animals with low ligation had a considerably higher, but also short-lasting, mean fibrinogen elevation. The highest values were encountered in animals with high ligation, thus tending to confirm a previous clinical report correlating the fibrinogen level with the severity of manifestations(1).

As seen from Fig. 1 fibrinogen levels in dogs are less and shorter lasting than in human myocardial infarction. Newly developed intercoronary arterial communications may produce more rapid recovery in the dog(8). It is also possible that dogs have a lesser fibrinogen response to injury than the human being.

Summary. 1. *In vitro* experiments indicate that the clot density determination of fibrinogen may be applicable to either human as well as canine plasma. 2. Fifty dogs were subjected to a) sham-operation, b) high coronary artery ligation, c) low coronary artery ligation. 3. Significant differences in the mean fibrinogen levels were found in the three groups corresponding to the various degrees of myocardial necrosis. 4. These results seem to bear out a previously reached clinical impression that the maximum fibrinogen concentration parallels the extent of the myocardial infarct.

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Urinary Levels of Mucoprotein Components in Normal Male Subjects.* (21784)

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(Introduced by Theodore E. Friedemann.)
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Recent studies(1-4) on normal human urine have shown that much of the high molecular weight material obtained by methods of precipitation consists of a mixture of carbohydrate and protein. Designation of this material as mucoproteins has seemed appropriate as a result of chemical analysis(4). Tamm and Horsfall(5) have isolated a homogeneous mucoprotein of enormous molecular weight(6) from urine by addition of NaCl. Other workers(7,8) have studied the electrophoretic distribution of urinary proteins in portions of the total colloidal material soluble in NaCl buffers.

Analysis of the total non-dialyzable material in normal human urine(9) has shown that about 80% of the total weight could be accounted for by carbohydrate components and protein. This paper will present the mean values of certain components of this high molecular weight material in urine. Determination of nitrogen, fucose, hexosamine, hexose and hexuronic acid were made on dialyzed aliquots of a 24-hour urine collection.

Materials and methods. Eight normal male laboratory personnel between the ages of 20 and 30 years served as subjects for this study. A single 24-hour urine was collected in polyethylene bottles with added thymol. The 24-hour volume was measured and an aliquot of 100 ml dialyzed for 3 days in running tap water at 10°C and then for 2 days in distilled water at 5°C that was changed twice daily. Analytical determinations were made in duplicate. For analysis of total hexosamine and hexose, 10 ml of dialyzed urine was concentrated to dryness in a wide bore test tube in a desiccator. Two ml of 4N HCl was added to the tube, care being taken to wash down the dried residue along the side. The tube was sealed and hydrolyzed for 6½ hours at 100°C. The hydrolysate was diluted to 25 ml with distilled water. Hexosamine and hexose were determined on aliquots of this hydrolysate—the former by a modified Elson and Morgan(10) procedure; the latter by the anthrone method as described by Scott and Melvin(11), using an equal mixture of galactose and mannose as standard sugars. Nitrogen was determined on 2 ml of dialyzed urine by a micro-Kjeldahl method(12). Fucose in dialyzed urine was determined by the method of Dische and Shettles(13) using a 3-minute boiling time. For the determination of hexuronic acid, preliminary hydrolysis of urine was necessary (14). For optimum yields 2 ml of dialyzed urine was hydrolyzed with 2 ml of concentrated HCl for 60 minutes at 100°C in pyrex test tubes with tapered joint stoppers with a 1 mm perforation.† A naphthoresorcinol method(15,16) was then used for the determination of hexuronic acid. Hexosamine and hexose were also determined in phosphotungstic acid precipitates of dialyzed urine. To 20 ml of urine was added one drop of 40% (w/v) potassium acetate and 5 ml of a 5% (w/v) phosphotungstic acid (PTA) solution in 2N HCl. After standing overnight at 5°C, the precipitate was centrifuged and the supernatant poured off. The precipitate was washed once with absolute ethanol. Two ml of 4N HCl was then added and the procedure described above for analyses of these substances followed.

Results. Table I summarizes the results of this study. The mean value of each determination is presented in terms of mg of the component excreted per 24 hours. The standard deviations of the values for the series of subjects as well as the standard deviations of the duplicate analyses(17) are listed. Table I

* The opinions expressed in this paper are those the authors and do not necessarily represent those of any governmental agency.

† Obtained from H. S. Martin & Co., Evanston, Ill.

TABLE I. Analyses of Mucoprotein Components in Dialyzed Aliquots of a 24-Hour Urine Collection from 8 Normal Males.

	Mean, mg/24-hr	Stand. dev. between subjects	Stand. dev. of single determination
Nitrogen	35.89	8.59	.96
Fucose	6.18	1.56	.16
Total hexosamine*	28.90	6.34	1.85
PTA† "	14.04	5.08	1.37
Total hexose*	38.43	9.04	2.92
PTA† "	14.14	4.90	1.26
Hexuronic acid	5.81	1.54	.33

* 7 subjects.
† Phosphotungstic acid precipitate.

also shows that PTA precipitates 48% of the total hexosamine and 36% of the total hexose; these substances exist in equimolar ratio in the precipitate.

Discussion. Studies similar to those on urinary mucoprotein components described in this paper are not generally available. Kerby (18) has reported on the levels of a high molecular weight glucuronic acid-containing component obtained by benzidine precipitation of urine. Normal males showed a mean 24-hour excretion of 4.9 mg glucuronic acid (determined by the carbazole method) in this fraction. Anderson and Maclagan(4) used the diphenylamine reagent in a colorimetric test with a mucoprotein fraction obtained from urine by adsorption on benzoic acid. Stern, Lillien, and Kagan(19) determined mucoprotein-tyrosine content of urine fractions soluble in perchloric acid but insoluble in PTA.

Studies(20,21) on the excretion of hexosamine and hexuronic acid in undialyzed urine cannot be compared with the data presented in this paper. The content of dialyzable hexuronic acid in urine is over 70 times that of hexuronic acid in the high molecular weight substance isolated by Kerby(18) and in dialyzed urine. The level of dialyzable hexosamine exceeds by 3- to 6-fold the content of non-dialyzable hexosamine in urine(22).

Summary. Analyses have been made of nitrogen, hexosamine, hexose, fucose, and hexuronic acid in dialyzed aliquots of a 24-hour urine collection from 8 normal male subjects.

The authors wish to acknowledge the aid of Dr. Morton Grossman with the statistical analysis.

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Determination, Recovery, Identification and Renal Clearance of Conjugated Adrenal Corticoids in Human Peripheral Blood.* (21785)

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Previous studies(1,2) indicated the presence of substances in human plasma presumed to represent corticosteroids conjugated as glucuronides. Because of the small amounts of material normally present in human plasma, it was not possible to identify the material released by enzyme hydrolysis with methods other than microchemical reactions and behavior on paper chromatograms. Due to the unavailability of "natural" corticosteroid conjugates, quantitative estimates of recovery were not reported. Experiments, herein described, were designed to elevate artificially the level of corticosteroids in human plasma in order to obtain larger quantities of steroids for identification. In addition the employment of the monoglucuronide of tetrahydro-E (pregnane-3α, 17α, 21-triol-11,20-dione-monoglucuronide) isolated from human urine and kindly donated by Doctor John J. Schneider, has permitted a study of recoveries. In the course of these studies, modifications of the method originally described(1) have presented certain advantages.

Methods. Plasma was added to 2.5 volumes of 95% ethanol within an hour of collection of a heparinized blood sample. Under these circumstances the sample may be stored for at least 48 hours at 10°C as noted below. The precipitate was removed either by centrifugation or filtration through Whatman no. 4 paper. The ethanolic solution was evaporated at 45°C with air, almost to dryness. A volume of water equal to that of the original volume of plasma was added to the residue and this extracted 3 times with ⅔ volume redistilled methylene chloride. The methylene chloride was evaporated to dryness and the residue chromatographed on florosil according to Nelson and Samuels(3). The latter contains the free corticosteroids which were measured by the Porter-Silber reaction as detailed below. The remaining *aqueous phase* was incubated at 37°C for 48 hours after the addition of acetate buffer (0.1M, pH 4.5) and 300-500 units beta-glucuronidase per ml. The latter was extracted as above with methylene chloride and the residue was chromatographed on florosil. This constituted the conjugated fraction. All *residues*. were treated as follows with appropriate blanks and standards. To each residue was added 0.25 ml absolute ethanol and 0.75 ml Porter-Silber reagent (65 mg phenylhydrazine hydrochloride in 100 ml 62% v/v sulfuric acid). These were left standing in the dark at room temperature for 16 hours. The resultant chromogens were read in the Beckman DU spectrophotometer at 370, 410 and 450 mu(3). In this laboratory the 17-hydroxy-alpha-ketolic steroids, Compounds E, F, S and their reduced tetrahydro derivatives, have been checked and all are equally chromogenic with

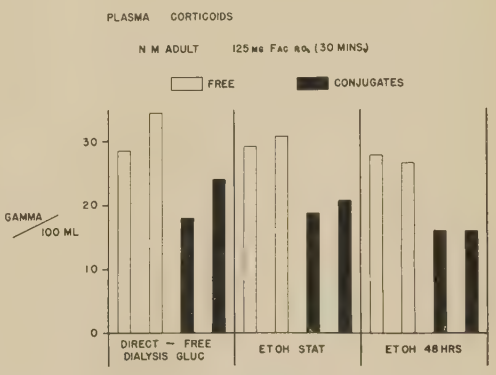

FIG. 1. Comparison of recovery of plasma corticoids following oral administration of Compound F between: (a) free : direct extraction vs ethanol precipitation with extraction at once and after 48 hr; (b) conjugates : glucuronidase hydrolysis of plasma followed by dialysis versus hydrolysis (after ethanol precipitation) at once and at 48 hr.

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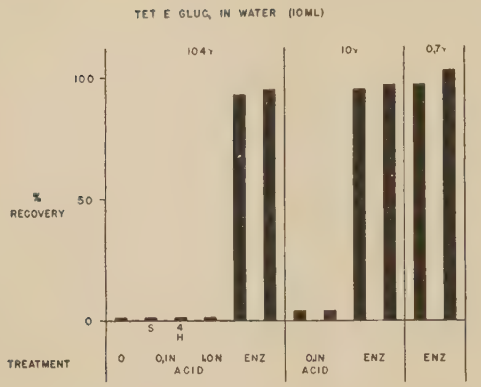

FIG. 2. Recovery of known quantities of monoglucuronide of tetrahydro-E from water following acid and beta-glucuronidase hydrolysis and direct extraction of the hydrolysate. 3,000 units of glucuronidase were employed for each 10 ml sample.

this procedure. Creatinine levels in blood and urine were determined according to the method of Hare(4).

Results. 1. *Comparison with previous method.* The above procedure has been compared with the more tedious method using dialysis previously reported(1). The results for the free and conjugated steroids so measured are illustrated in Fig. 1, wherein human plasma following the oral administration of Compound F was employed as the test material. The results between the two methods do not vary by more than 4% based on average values of duplicate specimens. When the material precipitated by ethanol is left standing at 10°C for 48 hours, the free corticosteroids show a loss not exceeding 10% and the conjugates, about 20%. There is less variation between duplicates with the method using ethanol precipitation than with the original procedure employing dialysis.

2. *Recoveries.* Pilot investigations were first conducted by addition of tetrahydro-E monoglucuronide to water (Fig. 2). Acid hydrolysis released no more than 5% of the material under all conditions studied: at pH 1.0 with immediate extraction or after 4 hours; or with 1.0 N hydrochloric acid. Hydrolysis with beta-glucuronidase released 93-102% of the material added to water at levels ranging from 0.7 to 104.0 per 10 ml. It was interesting that the monoglucuronide of tetrahydro-E was stable at 100°C for 10 minutes at pH 7.0 as determined by subsequent enzyme hydrolysis and the Porter-Silber reaction (Fig. 3). Again, treatment with hydrochloric acid at pH 1.0 room temperature or at 100°C released only insignificant quantities of material. The monoglucuronide of tetrahydro-E in water at pH 1.0 maintained at room temperature for 10 minutes or at 100°C for 10 minutes, was subsequently brought to pH 4.5 and hydrolyzed with beta glucuronidase; 76-95% of the added material was recovered. Human plasma stored under sterile conditions at 10°C for several days, was first directly extracted with methylene chloride. Various quantities of the relatively pure conjugate were then added to 10 ml aliquots and the samples were carried through the entire procedure as described. With quantities varying from 0.7 to 10.0 μg per 10 ml plasma the recoveries were 80-105% (Fig. 4). However when the amount added reached 100 μg, a condition not encountered under most clinical circumstances, the recovery was only 50%. Higher recoveries at this high plasma level were achieved when the plasma was exhaustively extracted (8-10X) with methylene chloride (see below). Recoveries from plasma with acid hydrolysis at pH 1.0 were never greater than 30% at any level,

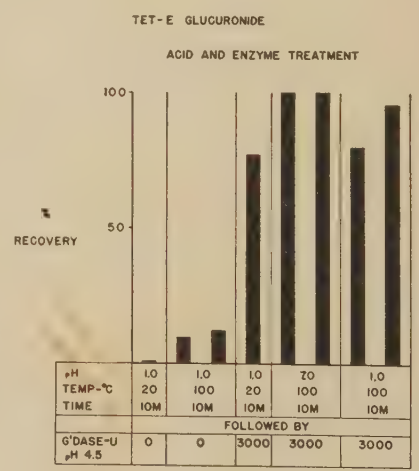

FIG. 3. Treatment of monoglucuronide of tetrahydro-E with heat, acid, and beta-glucuronidase. Indicates failure of hydrolysis with acid alone, and stability of compound with acid or elevated temperatures as measured by subsequent enzyme hydrolysis, where noted. In each case 107 μg were added to 10 ml water and measured as Porter-Silber chromogens.

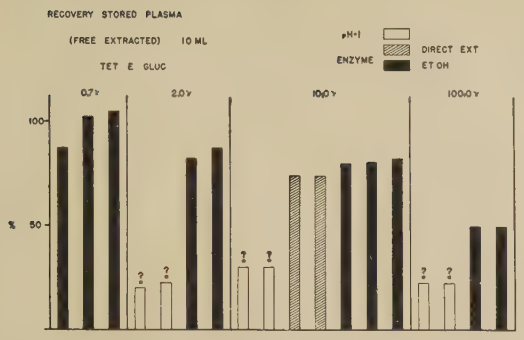

FIG. 4. Recovery of tetrahydro-E monoglucuronide added to human plasma in varying concentrations. The plasma employed had been stored for several days and contained no measurable quantities of corticoids prior to the addition of the standard.

and these were only apparent rather than real. Acid hydrolysis of plasma to which no compound had been added revealed values of the same order indicating the release of non-specific chromogens; this was confirmed by the failure of such material released in all experiments to show the usual maximum absorption at 410 mμ, although a correction factor was usually extractable, and such material subsequently submitted to paper chromatography failed to reveal substances resembling any of the corticosteroids. To determine the optimum concentration of beta-glucuronidase for the hydrolysis of conjugated corticosteroids, quantities of the enzyme varying from 1,000 to 15,000 units were added to 10 ml aliquots of human plasma containing 1.0 μg of conjugated Porter-Silber chromogens. Quantitative recoveries were obtained with 3,000-5,000 units of beta-glucuronidase per 10 ml of the aqueous phase. When the amount of enzyme exceeded 10,000 units the recoveries were apparently less (75%); this appeared to be due to the presence of large quantities of non-specific chromogens when excessive enzyme was employed.

3. *Identification of a single corticosteroid from the conjugated fraction.* To attain levels of corticosteroids in human plasma which would permit the isolation of large amounts of conjugates for definitive identification, large doses of tetrahydro-E (free alcohol) were administered in aqueous ethanol by mouth to a human subject in 8 doses of 250 mg each, every 30 minutes. In this experiment, the free corticosteroids were exhaustively extracted with volumes of methylene chloride equal to those of the aqueous phase 8-10 times before hydrolysis, and until the last extraction revealed no material measured as Porter-Silber chromogens. The remaining aqueous phase was then hydrolyzed with beta-glucuronidase and similarly extracted. The conjugated corticosteroids rose to levels heretofore unreported and exceeded greatly the quantities of free material as the experiment proceeded (Fig. 5).

Large volumes of blood were drawn after the sixth and eighth doses. The plasma was separated and treated as described. Aliquots of the compounds extracted after glucuronidase hydrolysis, were submitted to 70% methanol-hexane partitions, chromatographed on florisil and subsequently on paper according to systems modified after Bush(5,6). The mobility and reactions of the major component was identical to that of authentic tetrahydro-E. The substances eluted from the chromatogram gave quantitative results with the Porter-Silber and formaldehydogenic reactions, and sulfuric acid chromogens identical to tetrahydro-E. The melting point of the free unacetylated compound was determined: compound from plasma hydrolysate 197-198.5°C, authentic tetrahydro-E 198-

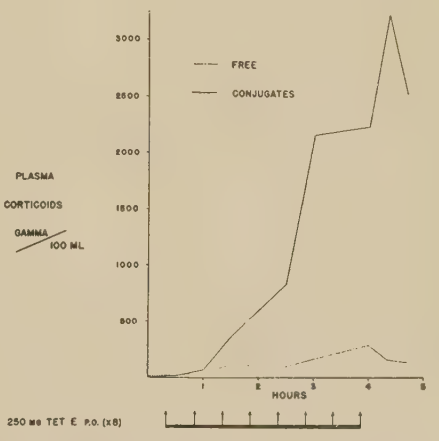

FIG. 5. Blood levels of free and conjugated corticoids during and after the oral administration of 8 doses of free tetrahydro-E, 250 mg each at 30 min. intervals to a human subject. The blood specimens were drawn 10 min. after each dose, in addition to a single control and 3 samples 10, 30, and 50 minutes after the final dose.

201, mixture 199-200. This substance was identified as tetrahydro-E by infra-red spectrophotometry through the kindness of Doctor Seymour Lieberman. The material extracted as the free component was similarly identified as tetrahydro-E by all the above criteria including infra-red spectroscopy.

In addition, a spot was isolated on the paper chromatograms from both the free and conjugated fractions of this same blood, which was stained by the nonspecific phosphomolybdate method (4% phosphomolybdic acid in absolute ethanol)(7). This reagent will detect a number of alcoholic and phenolic steroids without other functional groups, on paper. The spot did not react with triphenyltetrazolium on paper and did not give the Porter-Silber reaction but was formaldehydogenic. It migrated significantly *slower* than tetrahydro-E. It may represent one of the 20-reduced derivatives recently described by Fukushima(8), but has not as yet been sufficiently characterized.

The plasma proteins precipitated in the course of this experiment by the ethanol was saved and digested with Tryptar (Armour) at room temperature. The latter was extracted before and after hydrolysis with beta-glucuronidase. Chromatography of the residues on florisil followed by treatment with the phenylhydrazine reagent detected quantities of Porter-Silber chromogens which constituted only 1.2% of the total free fraction and 2.0% of the conjugates. These substances were not further characterized. It appears that only an insignificant portion of the corticosteroids remains bound to protein following employment of this method.

4. *Renal clearances.* In view of the high levels of free and conjugated corticosteroids achieved in the course of the oral administration of tetrahydro-E (Fig. 5), the renal clearance of each fraction was determined after the last dose was administered, during three 20 minute periods. (Table I). The free corticosteroids were cleared at an average rate of 11.2% and the conjugated 71.0% as compared to creatinine.

TABLE I. Renal Clearance of Free and Conjugated Corticoids after Oral Administration of Tetrahydro-E.

	Creatinine	Free	Glucuronide
ml plasma cleared/min. in three 20-min. periods	142	14.0	119.0
	130	16.9	75.2
	126	13.8	89.4
Avg	133	14.9	94.5
% related to creatinine		11.2	71.0

Clearances were run after administration of 8th dose (see Fig. 5).

Discussion. The method described for the extraction and measurement of both free and conjugated corticosteroids in blood has been tested and reveals certain advantages. The use of methylene chloride obviates the necessity of distillation immediately before use, as with chloroform(3). Methylene chloride has a lower boiling point and is easier to evaporate. The Porter-Silber reaction conducted at room temperature yields satisfactory chromogens for a large group of 17-hydroxy-corticosteroids. The use of the ethanol precipitation obviates emulsions and apparently preserves the corticosteroids in human plasma relatively well for 48 hours. It does not require dialysis.

The availability of the monoglucuronide of tetrahydro-E has made recovery studies feasible and within a wide range indicates that the method is reliable. The administration of large doses of tetrahydro-E (free alcohol) has resulted in the highest levels of conjugates yet reported in human plasma. The conjugates rose throughout this experiment to a degree greatly in excess of the free component, which justifies further attention to this fraction. As indicated earlier, in the face of markedly increased endogenous production of corticosteroids, the conjugated moiety may give a more accurate index of adrenocortical status than the free alone(1). This conclusion is admittedly based on an artificial experiment, which nonetheless serves to bring into greater focus, the extent to which rapid conjugation of corticoids in human plasma may limit the degree of rise of the free compounds.

Acid does not significantly hydrolyze the monoglucuronide conjugate in water, and only slightly so in human plasma. The material so released, cannot be characterized as adrenocortical steroids. The monoglucuronide of tetrahydro-E is completely stable at 100°C

for 10 minutes and pH 7.0, and slightly less stable at pH 1.0.

The definitive identification of a single steroid after the hydrolysis of human plasma with beta-glucuronidase supports the assumption that the "conjugated corticosteroids" measured in the manner described are circulating adrenocortical steroids. It is not possible to state the importance of protein-binding in the transport of corticosteroids from these experiments. However, following the ethanol precipitation of human plasma, only a small amount of steroids can be recovered from the digested protein precipitate.

The studies of the renal clearance of the free and conjugated steroids indicate that the latter are much more rapidly excreted than the former. It cannot be stated whether the difference in excretion of these two components is due entirely to a lower glomerular filtration rate of the free compounds or to differences in tubular reabsorption. It appears (Table I, Fig. 5) that the limited rise of the free corticosteroids in human plasma, in the course of the last experiment, is attributable more to mechanisms of conjugation than to renal excretion, although both may play a role.

Summary. 1. An alternative method for the extraction and quantitation of free and conjugated corticosteroids in human plasma has been described. 2. With the use of the relatively pure monoglucuronide of tetrahydro-E, it has been possible to show that the recovery of conjugated corticosteroids is satisfactory within a broad range of concentrations. 3. Following the administration of large oral doses of free tetrahydro-E, very high levels of conjugated corticosteroids, in excess of 3 mg/100 ml of plasma, have resulted. The material has been definitively identified as tetrahydro-E. A second component, possibly the 20-reduced derivative has been detected. 4. The renal clearances of the free and conjugated steroids in human plasma have been determined.

The authors are indebted to Doctor John J. Schneider for many suggestions, helpful criticism, and gifts of steroids. Doctor Seymour Lieberman has kindly performed the infra-red spectroscopic examinations. Doctor Wallace McCrory advised the authors on renal clearance studies. Mr. Frank Rosolia and Mr. Pasquale Pellecchia were responsible for technical assistance. The beta-glucuronidase employed, derived from beef liver (Ketodase, Warner-Chilcott), was generously donated by Doctor Raphael Cohen. The figures were drawn by Mr. Frank Rosolia.

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Influence of Hormones (Growth, ACTH, Thyrotrophic and Cortisone) Upon Complement Level in Rabbits.* (21786)

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Complement is thought to be involved in *in vivo* immunological reactions, but its exact role is uncertain(1). Attempts to alter complement by treatment with hormones known to affect certain disease states, the pathogenesis of which is presumed to involve antigen-antibody reactions, have been inconclusive (2). Among the few positive experiments are Cope's(3) report that thyroxine caused a small elevation and thyroidectomy a fall in serum complement of rabbits; Simonsen(4) reported that a single dose of cortisone caused a transient depression of complement in guinea pigs. Observations reported here indicate that levels of serum complement can, under certain conditions, be depressed by cortisone and elevated by growth hormone preparation administration.

Materials and methods. Animals. Seventy-five rabbits (Swift strain) were used weighing between 2 and 3 kg. They were housed in individual cages and fed a commercial ration ("Miracle") and water *ad lib.*

Serum preparation and storage. Animals were bled from the ear and serum removed from the retracted clot within 24 hours of bleeding. Sera were stored frozen at –20° to –25°C. *Complement titrations.* A 100% hemolysis end point was used in the system described by Kabat and Mayer(5). Aseptically drawn sheep's erythrocytes were stored in Alsever's solution. Two units of commercial rabbit hemolysin (Markham Co., Chicago) and veronal saline buffer with Ca^{++} and Mg^{++} were employed(6). Two dilution series were used for each serum, one starting at 1:2 and the other at 1:3. This provided replication and a closer endpoint approximation. Titrations of each week's sera were done separately, and at the end of the experiment selected sera from bleedings made at various times were retitered simultaneously. Maximum discrepancy between repeat determinations was 1 dilution and 70% of the replications yielded identical values. No significant deterioration could be discerned in complement titers of sera stored for four weeks or less at –20° to –25°C; after longer storage some reduction in complement activity was apparent. In the experiments described the values were measured within the 4 week interval.

Experimental observations. The effects of cortisone and growth hormone, alone and in combination are illustrated by the following experiment: A group of 20 rabbits, being used for studies of the effect of these hormones on antibody production(7), received 0.2 ml of a solution containing 0.5% bovine serum albumin and 0.5% bovine gamma globulin 5 days per week for 3 weeks. During the first week 10 animals were given 5 mg cortisone acetate (Schering) intramuscularly each day. The remaining 10 served as controls. All animals were bled on days 0 and 7. During the second and third week the 10 animals receiving cortisone were continued on that treatment and in addition 5 were given 10 mg of growth hormone† (Armour) intramuscularly each day; 5 of the control animals were likewise treated with growth hormone. Bleedings were repeated on the days 14 and 21.

Data on serum complement levels are recorded in Table I. The complement level of the control animals is relatively stable with a small tendency to rise. Animals treated with cortisone show a marked depression by the end of seven days which is sustained through the 21-day period. Animals treated with

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† Prepared after the method of A. Wilhelmi, and J. B. Fishman, given lot No. R491131B.

We are indebted to Dr. Irby Bunding of Armour and Co. Research Laboratories, for these materials and the information concerning their properties.

TABLE I. Effect of Cortisone and Growth Hormone on Complement during Immunization.

Rx	Days after initiation of therapy 0	7	14	21
	Serum complement levels†			
0*	10.6 ± 3.4	11.2 ± 1.5	12.0 ± .0	13.6 ± 1.0
Cortisone	10.8 ± 2.8	3.4 ± .6	3.9 ± 1.0	1.5 ± 1.5
Growth hormone (started day 7)	12.8 ± 1.5	10.4 ± 1.0	30.4 ± 9.1	18.5 ± 4.3
Cortisone + growth hormone (G.H. started day 7)	13.0 ± 3.8	4.4 ± .9	12.8 ± 3.2	10.0 ± 2.2

* All animals received antigen (albumin + glob.).

† Recorded as 100% hemolytic units/ml of serum; means and stand. errors; 5 animals per group.

growth hormone (started on the 8th day) show a marked rise in complement level at the end of one week's treatment (14th day of experiment) with this material. By the end of the experimental period there is some decline from the peak level. The superimposition of growth hormone therapy on previous cortisone therapy results in a rise of the previously depressed complement level to the normal range but no further rise.

In Table II are recorded the results of an experiment designed to test the following points: (1) Whether in the absence of antigen administration and hence circulating specific antibody cortisone therapy would cause a measurable decrease in complement levels. (2) Whether ACTH has a demonstrable effect on the complement level, and (3) whether thyrotropic hormone, a known contaminant of the growth hormone preparation used, would have any effect upon circulating complement. The total period of observation in these experiments was 8 days. Animals were bled prior to institution of therapy, and on days 1, 2, 4, and 8. Each treatment group was composed of 5 animals. The control group received no therapy. Cortisone and growth hormone were administered daily by the route and in the dose previously described. Thyrotrophic hormone (Armour) was administered in a dose of 2 mg per day intramuscularly. This dose is approximately equivalent to the quantity present as contaminant in 10 mg of the growth hormone preparation. The dose of ACTH was 5 mg equivalent (Armour Standard) per day intramuscularly. Data for the baseline, 4 and 8 day titers are recorded in Table II. Cortisone did not produce a significant drop in complement level in the absence of the immunization procedure, but there is no tendency of the complement level to rise, as seen in the controls. Growth hormone produced the same striking rise in complement as previously. Thyrotrophic hormone shows a similar, but slightly less marked rise. The complement level of the ACTH treated animals in this experiment is not significantly different from that of the controls.

TABLE II. Effect of Several Hormones on Complement in the Absence of Immunization.

Rx	Days 0	4	8
	Complement levels*		
0	13.2±3.2	16.0±4.8	15.8±6.3
Cortisone	13.4±3.6	13.6±3.5	9.6±2.4
Growth hormone	13.6±3.3	27.2±3.2	32.0±4.3
Thyrotropic hormone	8.4±2.0	19.2±4.8	23.6±6.0
Adrenocorticotropic hormone	9.2±2.2	18.4±4.3	17.6±4.7

* Means and stand. errors; 5 animals in each group.

Other trials of the combination of a course of antigen administration with administration of cortisone showed the depression of complement to be enhanced by the immunization. With ACTH, no significant, reproducible depression or rise of complement occurred when the drug was administered alone or in combination with antigen.

No anticomplementary action of the sera from cortisone treated animals on the complement activity of normal rabbit serum could be demonstrated.

Discussion. The data presented indicate clearly that cortisone has the capacity to depress complement levels in rabbits under certain circumstances. This effect is elicited best if small amounts of antigen are administered

into the circulation in the presence of small amount of circulating antibody. Other experiments in this laboratory(8) confirm this and help elucidate its mechanism. That others have not observed this(2) is perhaps because they were administering antigen in large doses and the complement diminution occurred in both cortisone and non-cortisone treated controls at about the time of appearance of circulating antibody.

ACTH had no apparent effect on complement; this is consistent with the findings of others(9). This difference in the effects of the two physiological related substances may be due to the fact that ACTH stimulation of the rabbit adrenal leads to increased production of corticosterone(10) and that the effect on complement is not mediated by this substance.

The effect of the growth hormone preparation is interesting and has, as far as we are aware, not been reported previously. Campbell(11) has found a rise in the levels of prothrombin and fibrinogen in animals treated with growth hormone. The experiments described here suggest that the action of the growth hormone preparation may be due to thyrotrophic hormone. This is consistent with Cope's(3) observations that following thyroidectomy the serum complement level falls.

The experiments presented indicate that cortisone and "growth hormone" in the rabbit have apparently opposing effects on complement. The interpretation of these alterations in relation to disease must await an understanding of the role of complement in normal and pathologic processes.

Summary and conclusions. Cortisone administration combined with administration of antigen in small doses intravenously causes significant and substantial decrease in circulating complement. Cortisone alone produces a hardly discernible effect. ACTH with or without simultaneous antigen administration has no apparent depressant action on complement. Growth hormone administered with or without simultaneous immunization produces a striking rise in the complement level. Evidence is presented suggesting that this may be due to the presence of contaminating thyrotrophic activity.

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Arginase Activity of the Salivary Glands and Its Regulation by Androgens.*† (21787)

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From Oklahoma Medical Research Foundation, Oklahoma City.

Androgens markedly influence the size of kidneys(1) and salivary glands(2). These phenomena are most readily seen in the mouse (1,3). Furthermore, androgens will alter the activity of several enzymes of the kidney(1). Arginase is one of the enzymes which is greatly influenced. It seemed worthwhile, therefore, to determine whether arginase was present in the salivary glands and, if so, whether androgens would produce a change in its activity similar to that observed in the kidney. This seemed of special interest since the presence of urea and ammonia in the saliva has been implicated in the incidence of dental caries(4).

Animals. The experimental animals were housed in air-conditioned rooms at a temperature of 25.5-26.6°C and the amount of artificial light regulated at 12 hours per day. The rats were purchased from the Sprague-Dawley Company and were maintained in individual cages. They were fed a prepared diet consisting of: Casein, 16.7; Yeast (Fleischmann's 2019), 9.2; Sucrose, 61.2; hydrogenated vegetable oil, 7.4; Cellu-flour, 1.8 and Wesson's Salts, 3.7. In addition, a daily supplement of one drop of Cod Liver Oil (Patch's) and one drop of a 34% tocopherol concentrate from wheat germ oil diluted ten-fold with Wesson oil was mixed with the food. The amount of food eaten was determined by weighing the food before and after feeding. The mice were purchased from the Stokley-Peterson Company and two to four were maintained in glass jars containing wood shavings. They were fed Rockland Rat Diet *ad libitum*. The animals were weighed 3 times per week. The testosterone propionate was a commercial preparation‡ in oil at 10 mg/ml. The thyroxine solution was prepared at 10-14 day intervals by dissolving 2.5 mg dl-thyroxine§ in 1 ml of 0.02 N NaOH and diluting with water to 25 ml. All injections were made at the same time each day. *Autopsy.* The rats were anesthetized with dial-urethane and bled to death. The mice were killed by severing the spinal cord with pressure. The salivary glands were removed and placed into 5 ml of cold, redistilled water. The tissues were homogenized with a Potter-Elvehjem type homogenizer and diluted to a concentration of either 1:50 or 1:100. One ml of this final homogenate was used for the determination of arginase activity by the method previously reported(5).

Results. Rats. Arginase activity was present in all 3 of the salivary glands (Tables I, II). The concentration of the enzyme in each of the salivary glands was essentially the same except that it was much less in the parotid glands of the older animals (Table II).

Castration (Table I) did not significantly alter the weight of the submaxillary or sublingual glands. The parotid glands were not removed quantitatively. The administration of testosterone propionate or methyl testosterone by subcutaneous implantation of pellets for a period of 22 days did not materially change the combined weight of the submaxillary and sublingual glands. Castration (Table I) did not produce a significant change in either the total units or the units per gram of arginase activity except in the older animals in which a difference which was significant at $P > 0.05$ was noted. The androgens produced a suggestive decrease in the units per gram of arginase activity towards

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† These data were presented at the meeting of the International Association of Dental Research on March 19, 1955.

‡ Generously supplied as perandren by Ciba Pharmaceutical Products, Inc.

§ Generously supplied in crystalline form by Roche-Organon.

TABLE I. Effect of Castration and Androgens on Arginase Activity of Salivary Glands of the Rat.*

Treatment	Rats No.	Body wt, g	Submaxillary and sublingual mg	Total U	U/g	Parotid,† U/g
			88 days old at autopsy			
Normal	6	315	373 ± 9‡	232 ± 7	626 ± 17	667 ± 12
Castrate	6	306	381 ± 19‡	271 ±115	701 ± 46	604 ± 30
			131 days old at autopsy			
Normal	5	382	481 ± 33	265 ± 20	552 ± 34	506 ± 30
Castrate	5	349	533 ± 22	367 ± 50	675 ±111	450 ± 38
" + T.P.§	5	356	592 ± 20	261 ± 38	447 ± 75	450 ± 37
" + M.T.§	5	338	545 ± 15	250 ± 45	421 ± 35	486 ± 52
			167 days old at autopsy			
Normal	5	406	523 ± 26	402 ± 18	759 ± 27	596 ± 24
Castrate	5	363	529 ± 21	510 ± 44	961 ± 51	623 ± 30

* Castration at 37 days of age. Values presented with stand. error of mean.
† Parotid gland was not removed quantitatively.
‡ Submaxillary glands only.
§ Testosterone propionate, 2 pellets, (0.37 mg/day) and methyltestosterone, 1 pellet (0.34 mg/day) implanted 22 days before autopsy.

that of normal but these values were not statistically significant. The administration of testosterone propionate at 0.5 mg/day for 60 days (Table II), however, produced definite changes in both the weight and the arginase activity of the submaxillary and sublingual glands. The weight of these 2 glands was significantly increased and the arginase activity, both as total units and U/g was markedly decreased. No changes were observed in the arginase activity of the parotid gland. The administration of thyroxine alone or in combination with testosterone propionate was ineffective on both the weight and arginase activity of the salivary glands.

Mice. The implantation of several different androgens as pellets in castrated mice produced within 21 days a marked increase in the weight of the submaxillary plus sublingual glands. The increases for the various hormones and at the different dose levels apparently were at a maximum response. The arginase activity in total units was slightly decreased but the differences were not statistically significant. The concentration of arginase activity, however, was decreased to at least a half and, in some instances, a third of that in the control animals. This decrease was roughly proportional to the increase in weight of these glands.

Discussion. The presence of arginase activity in the 3 salivary glands suggests that the urea present in the saliva is manufactured in these glands and not filtered from the blood. Arginase activity, however, occurs in a large number of tissues(6,7) and it is not clear as yet whether the activity in the various tissues is for the sole purpose of produc-

TABLE II. Influence of Thyroxine (Thy.) and Testosterone Propionate (T.P.) on Arginase Activity of Salivary Glands of Castrated Rats.*

Treatment	Submaxillary mg	Total U	U/g	Sublingual mg	Total U	U/g	Parotid,† U/g
Control	350 ± 20	193 ± 21	582 ± 57	220 ± 10	105 ± 8	478 ± 32	218 ± 31
Thyroxine, 5 μg	355 ± 17	209 ± 19	584 ± 34	240 ± 12	139 ±12	574 ± 58	206 ± 25
" 10 "	384 ± 3	164 ± 21	432 ± 64	249 ± 19	137 ±12	546 ± 34	249 ± 17
T.P., 0.5 mg	485 ± 36	116 ± 8	242 ± 14	282 ± 9	75 ± 5	265 ± 12	290 ± 14
Idem + Thy., 5 μg	435 ± 13	109 ± 10	252 ± 27	315 ± 14	78 ± 8	258 ± 25	310 ± 18
" + " 10 "	446 ± 20	117 ± 5	265 ± 15	313 ± 16	76 ± 7	242 ± 15	288 ± 19

* Castrated at 73 days of age. Treatment began 41 days later. Five rats/group.
† Parotid gland was not removed quantitatively.

TABLE III. Effect of Androgens on Weight and Arginase Activity of Salivary Glands of Castrated Mice.*

Steroid	Mice No.	Dose, mg/21 days	Fasting body wt, g	Submaxillary and sublingual		
				Wt, mg	Arginase — Total U	Arginase — U/g
Controls	12		34.6	141	43 ± 6.3	296 ± 38
Testosterone propionate	3	3.0	38.6	248	35	143
Testosterone	4	7.7	39.3	264	28	110
17-Methyltestosterone	5	5.5	36.8	235	32	138
"	3	9.5	39.3	258	30	124
Androstan-17β-ol,3-one	6	2.3	39.1	253	38	154
" " "	4	5.2	39.4	274	29	103
17-Methyl " "	6	3.1	39.6	279	28	100
" " "	4	5.4	38.5	266	31	114
Androstane-3α, 17β-diol	5	1.8	37.6	267	36	135
" " "	2	4.0	38.4	248	35	154
17-Methyl " "	4	2.5	37.3	237	32	134
" " "	4	5.9	34.8	249	30	120
4-Androstene-3, 17-dione	4	10.5	38.0	248	42	177

* Castration at 45 days of age (24-26 g body wt). Pellets (15 ± 1 mg) of androgens implanted 60 days later.

tion of urea(5).

The salivary glands, like the kidney, are excretory organs, furthermore, these glands respond to androgen treatment by an increase in weight comparable with that of the kidney. It is of interest, therefore, that the arginase activity of the salivary glands is decreased while that of the kidney is greatly increased. The arginase activity of the kidney is located primarily in the proximal convoluted tubules (8) which increase in size after androgen treatment(9,10). The excretory tubules of the salivary glands also increase in size(2). It would be of interest to know the site of arginase activity in the salivary glands. The portion(s) of the salivary glands which increases in size under the stimulus of androgen apparently is not that which contains arginase activity.

Summary. The salivary glands of rats and mice contain arginase activity. The activity of this enzyme in the submaxillary and sublingual glands of castrated rats was decreased only after prolonged treatment with androgens. The activity in the glands of the mouse, however, were readily decreased by a large number of C_{19} steroids. Thyroxine had no influence on the arginase activity of the salivary glands of the rat.

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Pharmacology of a Short-Acting Non-Sulphur Barbituric Acid Derivative (21788)

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The present investigation is concerned with a barbiturate identified by Lilly code number 22451, and having the formula 1-methyl-5-allyl-5-(1-methyl-2-pentynyl) barbituric acid, sodium salt, which shows considerable promise as an ultra-short acting barbituric acid.

Methods and specific compounds. Albino rats weighing 70 to 120 g (an average of 100 g) were used. Solution of the sodium salt of this compound was injected by vein. For each dose level, 8 rats or more were used to determine the anesthetic dose, duration of action, and lethal dose. Rabbits, dogs, and monkeys also were used. The median anesthetic dose ($AD_{50} \pm$ S.E.) and the median lethal dose ($LD_{50} \pm$ S.E.) were computed by the method devised by Bliss(1).

Results. Compound #22451 was compared with Pentothal and Thioseconal (Table I). It will be noted that the anesthetic dose of compound #22451 in rats was less than one-half that of Pentothal or Thioseconal. In rabbits the anesthetic dose of this compound was approximately one-fourth to one-fifth that of Pentothal or Thioseconal. Furthermore, in dogs the anesthetic dose of compound #22451 was approximately one-half that of Pentothal or Thioseconal. In monkeys the anesthetic dose of this compound was approximately one-third that of Pentothal or Thioseconal. The therapeutic index of all three compounds was approximately the same.

Table II represents the duration of action of compound #22451 in rats. In these animals #22451 was one-fourth to one-sixth as short-acting as Pentothal or Thioseconal. In rabbits the difference was one of approximately one-third; in dogs, about one-fifth; and in monkeys, one-half that of Pentothal or Thioseconal. Therefore, it is quite obvious that this compound is distinctly shorter acting than the two well known thio-barbiturates.

In dogs receiving the AD_{50} of compound #22451, the rectal temperature, pulse rate and respiratory rate were recorded. The response to this compound was a slight, very transitory fall of temperature, acceleration of heart rate and a slight decrease of respiratory rate.

In accordance with the method of Wyngaarden and his colleagues(4) the accumulative action of compound #22451 was determined and compared with Pentothal and Thioseconal. In our experiments approximately one-half of the AD_{50} was injected by vein in 8 or 12 dogs. The time that elapsed between injection and complete recovery was recorded. The injection was repeated every hour until the duration of action exceeded 60 minutes. The results are tabulated in Table III. After 8 hourly injections compound #22451 showed an average duration of action of less than 60 minutes in all dogs. In contrast, Pentothal had a duration of action greater than 60 minutes after the fourth injection, and Thioseconal after the sixth injection. If the time after

TABLE I. Comparison of AD_{50} and LD_{50} mg/kg by Intravenous Injection.

Compound	Rats		Rabbits	
	$AD_{50} \pm$ S.E.	$LD_{50} \pm$ S.E.	$AD_{50} \pm$ S.E.	$LD_{50} \pm$ S.E.
Pentothal(2)	29.0 ± 1.5	64.0 ± 3.0	23.0 ± 1.6	31.0 ± 2.21
Thioseconal(3)	33.0 ± 1.6	66.0 ± 2.0	18.1 ± 1.2	26.0 ± 1.4
#22451	10.74 ± .75	24.89 ± 1.74	4.74 ± .15	8.64 ± .28

Compound	Dogs		Monkeys,
	$AD_{50} \pm$ S.E.	$LD_{50} \pm$ S.E.	$AD_{50} \pm$ S.E.
Pentothal(2)	16.0 ± .97	36.0 ± 1.3	9.95 ± 1.39
Thioseconal(3)	13.3 ± .03	36.3 ± .83	10.23 ± .51
#22451	7.35 ± .50	21.5 ± 1.5	2.97 ± .22

TABLE II. Comparison of Mean Duration of Action in Minutes with Observed AD_{50} in mg/kg Dose by Vein Injection.

Compound	Rats		Rabbits		Dogs		Monkeys	
	AD_{50}	Duration	AD_{50}	Duration	AD_{50}	Duration	AD_{50}	Duration
Pentothal(2)	30	186	25	47	16	142	10.0	44
Thioseconal(3)	36	162	20	43	15	113	10.5	45.5
#22451	11.0	26	4.75	16.4	7.35	33	3	17

the first injection for each compound is taken as unity, and if the subsequent figures are expressed in per cent of the initial time, then it is clear that there is relatively less cumulation of action with compound #22451 than with Pentothal and Thioseconal.

In 4 dogs anesthetized with compound #22451 blood pressure and respiration were recorded. The vagus nerve was exposed to electric stimulation. Small doses of this barbiturate injected by vein caused a transient fall of blood pressure and increased pulse rate, accompanied by a decrease in both amplitude and rate of respiration. Furthermore, compound #22451 did not inhibit vagal response to electrical stimuli. Eight dogs were administered the anesthetic dose of #22451 daily for 4 weeks and no tolerance or sensitivity to the drug could be detected. At the end of 4 weeks the LD_{50} was administered to all 8 animals and proved to be lethal to 4 of the group. Like all short-acting barbiturates, #22451 lowered the blood pressure and depressed the respiration, especially when given rapidly by vein.

By intermittent intravenous administration of compound #22451 to 5 dogs for 3 hours, an average of 47.37 mg/kg was required to maintain anesthesia. At the end of 3 hours the injection was discontinued and the dogs were allowed to recover. The average recovery time was 107.2 minutes. The same procedure was repeated with Pentothal and Thioseconal; the former required an average of 50.1 mg/kg to maintain anesthesia and the latter, 48.5 mg/kg. Recovery time for both compounds was in excess of 4 hours. Thus with compound #22451 the dogs recovered from prolonged anesthesia more rapidly than with the two thio-barbiturates.

TABLE III. Comparison of Cumulation of Action per Hour by Vein Injection.

Compound	Pentothal	Thioseconal	#22451
Dose, mg/kg	8.0	6.5	3.75
No. of dogs	11	12	8
Hr	Mean duration of action, min. ± S.E.		
0	4.6 ± 1.8	5.2 ± .7	11.55 ± .46
1	9.7 ± 1.8	7.2 ± 1.6	12.67 ± .53
2	23.2 ± 4.8	10.2 ± 2.2	13.01 ± .63
3	66.3 ± 11.8	24.2 ± 5.5	14.08 ± 1.04
4	—	43.9 ± .6	15.29 ± .94
5	—	—	18.13 ± 1.78
6	—	—	17.97 ± 1.62
7	—	—	19.54 ± 1.92
8	—	—	22.57 ± 2.45

Table IV summarizes a study of the role of the liver and kidneys in sleeping time. The most significant increase in sleeping time occurred in animals with extensive liver damage produced by carbon tetrachloride. Those animals with partial (57%) hepatectomy presumably had sufficient functional liver tissue left to permit a more rapid recovery. The effect of nephrectomy is doubtful since there was no difference between nephrectomized and sham-operated animals. Obviously the liver has a major role in the detoxification of #22451.

TABLE IV. Effect of Liver Damage and Nephrectomy on Sleeping Times of Rats of No. 22451.

Group	No. of animals	Geometric mean ± S.E. of recovery times in min.
Control	10	15.68 ± 1.36
CCl_4 damaged	9	56.32 ± 13.18
Partially hepatectomized	18	25.35 ± 1.38
Nephrectomized	10	23.81 ± 1.59
Sham-operated	9	23.6 ± 1.7
Ether controls	10	18.2 ± 1.39

Summary. A new compound, 1-methyl-5-allyl-(1-methyl-2-pentynyl) barbituric acid, sodium salt, (#22451) has been studied and compared with Pentothal and Thioseconal in rats, rabbits, dogs and monkeys. It is a more potent anesthetic than either Pentothal or Thioseconal. With an observed AD_{60} this

compound has a shorter duration of anesthetic action than the two thio-compounds. When one-half of the AD_{50} is injected by vein at hourly intervals #22451 shows less cumulative action than Pentothal or Thioseconal. After prolonged anesthesia, recovery time is much more rapid with this agent than with Pentothal or Thioseconal and, as other experiments show, this transient action is related to detoxification by the liver.

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Lathyrus Factor Activity of Beta-Aminopropionitrile and Related Compounds.* (21789)

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Following the demonstration in this laboratory that β-(N-γ-L-glutamyl)-aminopropionitrile (I) is the natural causative agent of skeletal deformities produced in rats by *Lathyrus odoratus*(1,2), it was readily established that the toxicity was due to the beta-amino-propionitrile (BAPN) portion of I. The lathyrus activity of BAPN has also been observed independently by others(3,4). Knowing the formula of the toxic component, it seemed desirable to study further the mechanism by which BAPN exerts such a profound effect on mesenchymal tissues in growing rats. It has been demonstrated that casein in the diet has a modifying influence upon the severity of skeletal deformity(5) and incidence of spontaneous aortic rupture (6) following the feeding of crude sweet pea meal to growing rats. Because of this, in experiment A certain supplements were selected at random and fed with BAPN to ascertain whether they might influence the development of lathyrism.

Exp. A. Seventy-seven Sprague-Dawley rats of both sexes weighing 37-45 g were used in separate assays. These animals were fed one of the following diets up to 72 days.

Diet	A	B	C
Crude casein (Bordens)	10	21.5	12
Dried brewer's yeast (Schlitz)	20	10	—
Cerelose	63	61.5	81

All 3 diets also contained 4% Wesson salt mixture and 3% olive oil containing fat-soluble vitamins sufficient to provide the following amounts per kg of diet: 1850 I U vit. A acetate, 5.9 I U vit. D, 10 mg α-tocopherol, and 1.2 mg vit. K. In diet C pure vitamins in concentrations of 0.5 mg folic acid, 0.5 mg biotin, 2.5 mg pyridoxine hydrochloride, 2.5 mg thiamine hydrochloride, 3 mg riboflavin, 10 mg nicotinic acid, 20 mg calcium pantothenate, 1 mg i-inositol, supplemented with 1 g each of choline, L-cystine, and DL-methionine, per kg of diet were substituted for the brewer's yeast. In 7 groups, assays for toxicity were done either with BAPN alone or in combination with one of the following: 0.30 and 0.35% choline, 0.86% methionine, 0.35%

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TABLE I. Effect of BAPN and Supplements in Growing Rats.

Assay group	Diet	BAPN, %	Supplement, %	No. of rats	Days on diet	Wt gain and incidence of changes: g/day	Fibrosis femur	Paralysis hind limb	Aortic rupture	Hernia
1	A	.0	.0	8 (0)†	50	1.57	—	—	—	—
2	A	.2	.3 Choline	6 (6)	23-51	.88	6	—	1	3
3	A	.2	.86 Methionine	6 (5)	25-51	1.04	6	—	3	—
4*	B	.35	.35 β-alanine	10 (9)	20-72	.94	10	3	2	1
5	B	.35	.35 Choline	10 (8)	17-72	.56	10	—	—	—
6	C	.0	.0	6 (0)	49	1.60	—	—	—	—
7	C	.2	.0	12 (3)	30	1.45	12	2	2	5
8	C	.2	2.0 Cholesterol	9 (0)	47	1.51	9	—	—	2
9	C	.2	.7 L-arginine 1.4 L-lysine	5 (0)	40	1.11	5	—	—	—

* See Group 3, Table II for dietary control.
† No. of rats that died during experiment is shown in parenthesis.

β-alanine, 2.0% cholesterol, and a mixture of 1.4% L-lysine and 0.7% L-arginine. The animals were weighed weekly, and autopsied at the time of death or at the termination of the experiment. Such alterations as skeletal deformities, malformation of the femurs, hind limb paralysis, hernias, and spontaneous aortic ruptures were recorded.

Results. In the 2 dietary control groups (No. 1 and 6) weight gain was poor due to the low casein concentration. None of the control groups developed any abnormalities of bone. Irrespective of the supplement which was combined with BAPN (No. 2-5, 8, 9) skeletal deformities and osteoporosis were observed in every instance. Even though it was not possible to inhibit periosteal fibrosis of the femur or skeletal deformity, there is a suggestion that other related changes such as herniation, paralysis, aortic rupture, weight gain, and muscle spasticity may have been affected by the supplements. For example, hernias were not observed when the diets were supplemented with methionine, or a mixture of L-arginine and L-lysine (Assays 3,9). Hind limb paralysis, secondary to marked vertebral column deformities, was most pronounced in the group which received 0.35% BAPN and 0.35% β-alanine (Assay 4). Aortic rupture was seen in rats receiving BAPN alone (No. 7) and when supplements were added (No. 2, 3, 4). When L-arginine and L-lysine were fed with BAPN (No. 9), even though the weight gain was not as great as in No. 7, neither herniation nor paralysis was encountered. Feeding 2.0% cholesterol along with BAPN did not prevent malformation of bone (Assay 8). It is therefore possible by the administration of the synthetic BAPN to produce all of the changes in growing rats that result from feeding the natural sweet pea (*Lathyrus odoratus*) meal. A consistent observation not apparent in the Table is that the periosteal fibrous proliferation of the femur is more marked after 0.2 or 0.35% BAPN than after feeding 50% crude sweet pea meal.

Exp. B. Substances chemically related to BAPN were assayed for toxicity to investigate the relation between structure and biologic activity. Twelve groups of rats were used. Three were fed basal diets without addition of test substances. Two received BAPN, and the other 7 were given various compounds chemically related to BAPN.

In the dietary control rats, (No. 1, 3, 8) weight gain was poor and skeletal abnormalities were not observed. When 0.2% BAPN was added to diet A, weight gain was decreased, skeletal deformities were severe and 4 of 8 rats developed hernias (No. 2). Addition of 0.15% BAPN to diet B produced femoral fibrosis in all and hind limb paralysis in 1 (No. 4). Three analogs of BAPN, ethylene cyanohydrin, β-methylamino propionitrile, and β-dimethylamino propionitrile when added at a 0.35% concentration to diet B did not produce any observable skeletal alterations (No. 5, 6, 7). The only unusual alteration was the presence of bronchopneumonia in 3 rats in 2 of these 3 groups. The assays indicate that when a hydroxy is substituted

TABLE II. Assays of BAPN Analogs in Growing Rats.

Assay group	Diet	Chemical added, %	No. of rats	Days fed	Wt gain and incidence of lesions			Misc.
					g/day	Fibrosis femur	Vertebral col. deformity	
1	A	Control	8 (0)*	50	1.57	—	—	—
2	A	.20 $NH_2CH_2CH_2CN$	8 (8)	43-67	.50	8	7	Hernia 4
3	B	Control	5 (2)	55-64	1.45	—	—	Pneumonia 5
4	B	.15 $NH_2CH_2CH_2CN$	5 (0)	55	2.03	5	2	Paralysis of hind limbs 1
5	B	.35 $HOCH_2CH_2CN$	6 (2)	52-56	1.31	—	—	—
6	B	.35 $CH_3NHCH_2CH_2CN$	6 (0)	62	1.85	—	—	Pneumonia 2
7	B	.35 $(CH_3)_2NCH_2CH_2CN$	6 (0)	56	1.68	—	—	" 1
8	C	Control	5 (0)	49	1.60	—	—	" 1
9	C	.30 HCl $NH_2CH_2CH_2CN$	11 (9)	36-49	.73	11	7	Hernia 3
10	C	.30 $NH(CH_2CH_2CN)_2$	11 (1)	45-49	.44	11	—	Cerebellar incoordination 11
11	C	.30 $CH_3CONHCH_2CH_2CN$	11 (6)	44-49	.79	—	—	Pneumonia 7
12	C	$NH_2(CH_2)_3NH_2$	11 (0)	45	1.38	—	—	—

* No. of rats that died during the experiment is shown in parenthesis.

for an amino group in BAPN, there is a loss of biologic activity. Furthermore if one or 2 methyl groups are incorporated on the amino group there is also a loss of activity. This indicates that lathyrism is not due solely to the presence of a nitrile group in BAPN, and that the unsubstituted amino group is essential for lathyrus activity. The hydrochloride salt of BAPN is just as effective in producing lathyrism as the liquid free base (No. 2, 9).

Bis-β-(cyanoethyl)-amine at a 0.3% concentration produced nervous symptoms suggestive of cerebellar incoordination within 2 weeks (No. 10). The rats developed muscle spasticity and weakness of the extremities with an unsteady gait, and turned in circles. The weight gain was poor and sternal deformities became evident in 3 of 11 rats after the 5th week. At autopsy there was minimal to moderate proximal exotosis and periosteal fibrosis as well as increased shaft diameter of the femur. Kyphosis or scoliosis of the vertebral columns did not occur which is in contrast to the results in Assays 2 and 9. Bis-β-(cyanoethyl)-amine unlike BAPN produced early symptoms of central nervous system disturbance and did not exert so great an influence on bone development. Microscopic examination of the brain and spinal cord from 3 rats in this group revealed hydropic degeneration or necrosis of the Purkinje cells in the cerebellum and the ganglion cells of the anterior horn.

The rats fed 0.3% acetyl BAPN did not gain weight after the 4th week and 7 of 11 animals developed bronchopneumonia before death. Bony deformity was not observed in this group of rats. Trimethylene diamine at a 0.3% concentration did not produce lathyrism. Thus when the nitrile is replaced by an amino group there is loss of biologic activity. This suggests that even though the nitrile group alone is incapable of producing lathyrism, the nitrile is essential in the molecule for the production of mesenchymal changes in growing rats.

Discussion. It has been recently reported that aminoacetonitrile, BAPN and Bis-β-(cyanoethyl)-amine all produce lathyrism(4). We have observed similar effects on skeletal development with 2 of the above mentioned compounds. The previous workers did not describe any nervous symptoms following the feeding of Bis-β-(cyanoethyl)-amine which is in contrast to our observations. The influence of Bis-β-(cyanoethyl)-amine on nervous tissue is of considerable interest because this observation may have a bearing on the problem of toxic neurologic activity produced by some species of peas which has been reported by Lewis(7). Our studies indicate that the ability of an organic nitrile to produce lathyrism is greatly influenced by the presence

of a reactive amino group. The mechanism whereby BAPN exerts its influence on mesenchymal tissue is not apparent from these studies.

Summary. β-amino propionitrile will produce all the changes which have been described in rats fed 50% *Lathyrus odoratus* pea meal. None of the supplements fed with BAPN inhibited the development of skeletal deformities. Substitution of a methyl or acetyl into the amino group or a substitution of hydroxy for the amino group in BAPN results in a loss of biologic activity. Unlike BAPN which principally modifies bone development and produces marked skeletal deformity, Bis-β-(cyanoethyl)-amine exerts an early and pronounced influence on the central nervous system and subsequently produces lesser skeletal alterations.

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Anaphylaxis and Histamine Shock in Mice. (21790)

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Since 1948(7), when we made the unexpected observation that mice injected with *Hemophilus pertussis* vaccine developed sensitivity to histamine, considerable literature has been published on this phenomenon. Apparently a marked difference exists among various strains of mice in sensitization to bacterial antigens. Our observations, described below, deal with the comparison of anaphylactic and histamine sensitivity in different strains of mice after inoculation by *H. pertussis* vaccine.

Materials and methods. The technic of sensitization of mice by injection of *H. pertussis* vaccine has been described in many publications by us and others(2,7,11). In the present communication are summarized our studies with 3 strains of mice: CFW strain, from Carworth Farms, and 2 strains from Millerton Research Farms: hybrids C-58 F-1 and Princeton. The mice were kept for several weeks in this laboratory. In all cases we used young females of about 20 g body weight, and sensitized them by injection of *H. pertussis* vaccine: in some, 15 billion cells, in others 30 billion. Larger doses of vaccine were tried on the strains of mice resistant to histamine sensitization. On the

TABLE I. Effect of Vaccination of Different Strains of Mice with *H. pertussis* Vaccine on Sensitivity to Histamine (No. of Dead Mice in Numerator, Total No. in Denominator).

Dose of histamine diphosphate, mg/kg	Histamine shock in sensitized mice			Resistance to histamine
	Princeton	Hybrids C-58, F-1	CFW (Carworth)	Normal CFW not vaccinated
50	0/1		10/10	0/4
100	0/3			2/9
200	0/7	0/2	3/3	6/20
500	0/2	0/2	1/1	0/8
800	0/2	0/5	3/3	0/7
2000			3/3	0/5
Total	0/15	0/9	20/20	8/53

* This investigation was supported (in part) by research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.

TABLE II. Effect of *H. pertussis* Nucleoprotein on Mice.

Dose	Princeton	Hybrids C-58, F-1	CFW (Carworth)
	Anaphylactic shock in sensitized mice		
A. Shocking dose of nucleoprotein containing 0.1 mg N	10/15	9/12	39/44
	Effect on normal mice (not vaccinated)		
B. *Idem*	0/6	0/5	0/6

fifth day after administration of vaccine the mice were tested, some by injection with histamine, others with antigen. As an antigen we used *H. pertussis* nucleoprotein as described earlier(5). For histamine shock, mice were injected with different amounts of histamine diphosphate. For anaphylaxis, nucleoprotein was injected in a dose containing 0.1 mg nitrogen. All injections were made intraperitoneally.

Results. After vaccination with 15 billion cells of *H. pertussis* vaccine, the mice of CFW strain developed a high sensitivity to histamine and showed 100% mortality; only a few normal mice, CFW strain (extreme right column in Table I) of the same age as sensitized animals succumbed from similar doses of histamine; 45 out of 53 survived. The mice of the 2 other strains—hybrids C-58 F-1 and Princeton—injected with 15 and 30 billion cells of vaccine, did not show an increase of sensitivity to histamine (Table I). However, all 3 strains developed sensitivity to the nucleoprotein, and a high mortality resulted from anaphylactic shock.

Discussion. Although it has usually been considered difficult to produce anaphylaxis in the mouse, Fink and Rothlauf(1) described the symptoms of hypersensitivity in different strains of mice sensitized to egg albumin. Munoz and Schuchardt found among various strains of mice considerable differences in the degrees of sensitization to histamine after inoculation with *H. pertussis* vaccine(4). Unfortunately, these two authors worked only with young mice 14-16 g weight and 3-6 weeks old, and did not evaluate the significance of aging on the development of hypersensitivity. For this reason they incorrectly described the CFW strain as resistant to sensitization to histamine. Kind(2) was the first to stress the effect of aging on the development of sensitivity to *H. pertussis* vaccine.

Pittman and Germuth(11) published very precise quantitative studies on passive anaphylaxis with antibovine rabbit serum in mice inoculated with *H. pertussis* vaccine. Solotorovsky and Winstein(12) actively sensitized mice by alum precipitated bovine albumin and were able to protect them against anaphylaxis by pre-treatment with cortisone. Kind and Parfentjev(3) performed preliminary experiments with the protection by cortisone of mice sensitized by *H. pertussis* vaccine. In our studies genetic differences in mice were apparent. All three strains inoculated with *H. pertussis* vaccine developed sensitivity to antigen (*H. pertussis* nucleoprotein) and only the CFW strain became sensitive to histamine. In current experiments we used mice of 20-22 g body weight and 2-3 months old. It is difficult to sensitize to histamine very young mice (about 10 g body weight) of the CFW strain by injection of *H. pertussis* vaccine(6). Normal mice of 20-22 g body weight tolerated 2,000 mg histamine diphosphate per kilo but, after inoculation with *H. pertussis* vaccine, the median lethal dose for these mice was reduced to 25 mg histamine(10). Old CFW mice normally develop a high sensitivity to histamine. Many old ex-breeders, above one year of age and about 35 g body weight, died from the injection of 25 mg histamine per kilo without preliminary sensitization by *H. pertussis* vaccine. In the same strain, sensitivity to histamine can be induced by factors other than vaccination. For instance, transplantation of sarcoma 180 increased susceptibility to histamine, although not to such a high degree as did immunization by *H. pertussis* vaccine(8).

In addition, it was possible to show that an anaphylactic reaction can be distinguished from histamine sensitivity. For instance, in-

jection of certain fractions of yeast protein in mice sensitized by *H. pertussis* vaccine reduces their susceptibility to antigen, *i.e.* nucleoprotein, but not to histamine(9). On the contrary, treatment of vaccinated mice with antihistamine inhibits the sensitivity to histamine and, in lessened degree, to antigen(10). The information presented indicates that CFW mice represent a strain predisposed to histamine sensitivity, which normally develops in old animals, but which can be provoked earlier and in a higher degree in young adult animals by vaccination with *H. pertussis* and transplantation of sarcoma 180.

Summary. After the injection of *H. pertussis* vaccine, according to the genetic differences of mice, some strains of mice develop sensitivity to antigen, others to both antigen and histamine.

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Metabolic Comparison of Particulate Components of Rat Liver Cytoplasm.* (21791)

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Differential centrifugation of rat liver homogenate, suspended in either 0.25 M or 0.88 M sucrose, yields 2 layers in the mitochondrial fraction. Upon resuspension and recentrifugation of this fraction in a sucrose medium, there is formed a lower, yellow layer and an upper, pink layer. The nature of the pink layer is controversial. Jackson, Walker and Pace(1) have offered evidence that this material consists largely of microsomes. Siekevitz(2) believed it to be a mixture of microsomes and mitochondria and discarded it in his work on the relation of microsomes and mitochondria to protein biosynthesis. On the other hand, Kuff and Schneider(3) listed the pink layer as the upper 2 layers of the 6 mitochondrial fractions isolated by them. The material was found to have succinic dehydrogenase activity, which is characteristic of mitochondria and not of microsomes. This was confirmed by Laird, Nygaard and Ris (4), who determined that the succinic dehydrogenase activity relative to the protein nitrogen of the upper mitochondrial layer was 80% of that of the lower mitochondrial fraction. Laird and coworkers(5) suggested that this upper fluffy pink layer consists of a distinct type of mitochondria on the basis of the difference in size, and such properties as the ribonuclease to protein nitrogen ratio and alkaline phosphatase activity from that of the lower layer, the normal mitochondria.

In connection with an investigation of the role of the cytoplasmic constituents in protein synthesis, a comparison was made of certain metabolic properties of the pink mitochondrial material with that of the yellow mitochondria and microsome fractions.

Methods. Isolation of cytoplasmic frac-

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TABLE I. Specific Activity of the Protein of 4 Cytoplasmic Fractions.*

Substrate	Cytoplasmic fraction	Specific activity, cpm/mg protein	% radioactivity of microsomes
DL-Phenylalanine-3-C^{14}†	Microsome	620	—
	Mitochondria-P	306	49.3
	" -Y	112	18.1
	Supernatant	183	29.5
DL-Serine-3-C^{14}‡	Microsome	560	—
	Mitochondria-P	340	54.3
	" -Y	184	32.9
	Supernatant	236	42.1
DL-Lysine-2-C^{14}§	Microsome	349	—
	Mitochondria-P	188	53.9
	" -Y	89	25.5
	Supernatant	90	25.8
Na acetate-2-C^{14}‖ and Na acetate-1-C^{14}	Microsome	342	—
	Mitochondria-P	298	87.2
	" -Y	106	31.0
	Supernatant	190	55.5

* The substrates were injected, I.P., into male, Long-Evans rats. Livers were excised, homogenized and cell cytoplasm separated into 4 purified fractions by differential centrifugation. Protein from these fractions was precipitated with 10% TCA, washed, plated and counted to determine their specific activity.

† Two 0.25 ml of aqueous solution containing 6 mg/ml (1.5 μc/mg) injected into two 150 g rats at 25 min. intervals. Rats sacrificed 30 min. after second injection.

‡ Four injections of 0.25 ml of solution containing 2 mg/ml (32 μc/mg) given at 15 min. intervals into two 140 g rats. Animals sacrificed 90 min. after first injection.

§ Four injections of 0.25 ml of solution containing 1 mg/ml (1.5 μc/mg) given at 15 min. intervals to 170 g rat. Animal sacrificed 90 min. after first injection.

‖ Three injections each alternately of 0.25 ml of solution of carboxyl and C_2-labeled acetate given to two 160 g rats. Acetate solutions contained 5.5 mg/ml (12 to 14 μc/mg) of radioactive compounds. Animals sacrificed 90 min. after first injection.

tions. This was accomplished by differential centrifugation according to the procedure given in Table I of Hoster and coworkers(6). The rats were sacrificed by decapitation and the livers quickly excised, weighed, and washed in 0.88 M sucrose. They were then finely cut up with scissors and homogenized with 5 volumes of 0.88 M sucrose for 2 to 2.5 minutes in a Potter-Elvehjem all glass homogenizer cooled in an ice bath. The centrifugation was then carried out according to the scheme shown in the Diagram. The first 3 centrifugations were done in an International Refrigerated Centrifuge, all subsequent ones in the Spinco preparative ultracentrifuge.

Determination of radioactivity. Protein was isolated and treated according to the method of Peterson and Greenberg(7) in preparation for counting. The counting was carried out in a Tracerlab gas flow Geiger-Müller counter.

Results. Incorporation of amino acids in vivo. The incorporation of radioactive amino acids into the proteins of the 4 cytoplasmic fractions of the liver after intraperitoneal administration of C^{14}-labeled phenylalanine, serine, lysine and acetate is shown in Table I, showing that the specific activity of the microsome protein is about twice that of the mitochondria-P protein for the 3 different amino acids. With labeled acetate the specific activity ratio of microsome to mitochondria-P protein was approximately unity. The table also shows that the accepted mitochondrial fraction (mitochondria-Y) was the least active of the 4 in its uptake of amino acids.

An *in vitro* incubation of the 4 isolated fractions with DL-phenylalanine-3-C^{14} gave the figures for specific activity of: microsomes, 170; mitochondria-P, 254; mitochon-

Scheme for Isolation of Cellular Fractions by Differential Centrifugation

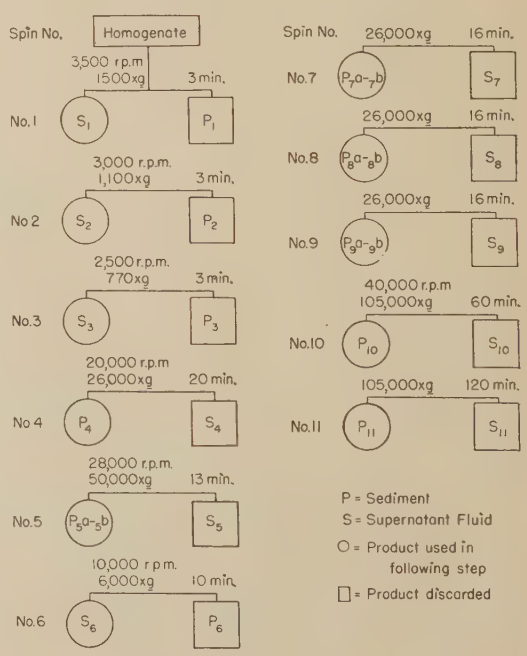

TABLE II. Radioactivity of the Non-Protein Material from Cytoplasmic Fractions.*

Substrate	Cytoplasmic fraction	Total radio-activity (cpm)	% of total radio-activity
DL-Phenylalanine-3-C^{14}	Microsomes	299	9
	Mitochondria-P	180	6
	" -Y	114	4
	Supernatant	2,596	81
DL-Serine-3-C^{14}	Microsomes	1,032	3
	Mitochondria-P	480	2
	" -Y	440	1
	Supernatant	31,900	94
DL-Lysine-α-C^{14}	Microsomes	735	9
	Mitochondria-P	475	6
	" -Y	345	4
	Supernatant	6,834	81
Na acetate-α-C^{14} and Na acetate-1-C^{14}	Microsomes	598	2
	Mitochondria-P	350	1
	" -Y	477	1
	Supernatant	37,600	96

* When the cytoplasm has been separated into 4 fractions, the protein was removed with 10% TCA. An aliquot of the TCA washings from each component was evaporated to dryness and counted to determine the specific radioactivity of the non-protein fractions.

dria-Y, 60; and supernatant liquid, 2.

The observed incorporation activity of microsomes, mitochondria and supernatant proteins are in agreement with the findings of Borsook *et al.*(8), Keller(9) and Hultin(10). The radioactivities of the non-protein material from the cytoplasmic fractions are recorded in Table II. Most of this was in the supernatant fluid.

Ratios of nucleic acid to protein radioactivity. Since the β-carbon of serine is utilized to form carbons 2 and 8 of the purines and the methyl group of thymine, it was possible to obtain some information on the relative activities of nucleic acid and protein in incorporating the labeled carbon of this amino acid. The nucleic acid and the residual protein were purified and their radioactivity determined. The ratios of nucleic acid to protein radioactivity for the 4 cytoplasmic fractions are given in Table III. The results show a distinct difference only for the microsomes in which the ratio was about ½ that of the other 3 fractions.

Cholesterol. With acetate, which is an excellent precursor of cholesterol, a comparison was made of the radioactivity of the cholesterol in the different fractions. The data (Table IV) show approximately equivalent specific activities in microsomes and mitochondria-P and in mitochondria-Y and supernatant liquid.

Effect of Aging upon Proportion of Y to P Mitochondria. In the course of our work it was noted that the proportional amounts of mitochondria–P and –Y varied with the age of the rats employed and this was investigated in greater detail.

Non-fasted groups of male rats of the Long-Evans strain from 13 up to 426 days of age were used for the test. Six grams ($\pm$ 0.1 g) of liver were employed whenever possible, and where not, the results are given on this basis. After separating the 2 mitochondrial fractions, the protein was obtained free of nucleic acid and lipides as previously described (7) and was then dried and weighed.

The procedure for the isolation of each particle was standardized so that all losses due to the separations would nearly cancel each other out, and great pains were taken to obtain complete separations without discarding any portion of the mitochondrial fractions. The isolations of the two mitochondria fractions are considered to be semiquantitative. The amount of protein from them which remained undifferentiated was approximately 5% of the total weight of protein isolated.

The results plotted in Fig. 1 show that there is a steady increase in the ratio of the protein of the mitochondria–Y to mitochondria–P with increasing age of the rats. From

TABLE III. Ratios of Nucleic Acid to Protein Radioactivity for 4 Cytoplasmic Fractions after Injection of DL-Serine-3-C^{14}.*

Cytoplasmic fraction	Nucleic/protein radioactivity (cpm)
Microsomes	.8
Mitochondria-P	1.9
" -Y	1.5
Supernatant	1.4

* After protein and nucleoprotein of the 4 fractions were precipitated with 10% TCA and resuspended in 5% TCA to remove extraneous radioactivity, they were heated at 90° for 15 min. in 5% TCA to remove the nucleic acid moiety. The remaining protein was further purified and hydrolyzed. A 0.2 ml aliquot of both substances was plated and counted and the ratio of their total radioactivity was calculated.

TABLE IV. Specific Activity of Cholesterol from Acetate-C^{14}-Cytoplasmic Fractions and Blood.*

Cytoplasmic fractions	Specific activity (cpm/mg cholesterol)
Microsomes	2492
Mitochondria-P	2866
" -Y	1816
Supernatant	1920
Blood	769

* Cholesterol was extracted from the lipid solvent washings of the protein from the 4 cytoplasmic fractions. It was precipitated as the digitonide, plated, weighed and counted to determine the specific activity.

the data of Table I it may be assumed that this, also, is equivalent to a decrease in the capacity for the rate of amino acid incorporation into protein with age per unit weight of liver, since mitochondria-P is much the more active in this respect. Since the weight of the liver increases with age it compensates for the drop. Fig. 1 also shows that the decrease in the growth rate of the animals coincides well with the curve for the mitochondria–Y to mitochondria–P ratios.

The total protein in the 2 mitochondrial fractions was fairly constant, varying only between 212 to 258 mg per 6 g of liver, but with age the protein of mitochondria–P decreased while that of mitochondria–Y increased correspondingly. Since the mitochondria–Y appears to be metabolically less active, there appears to be some association between this observation and the decrease in metabolic activity upon aging.

Discussion. The pink mitochondrial fraction may be a type of microsome, a type of mitochondria, a mixture of the 2, or a distinctly different cytoplasmic particle, with its own characteristic properties. The possibility that it is a mixture of microsomes and mitochondria is minimized by the observation that upon resuspending the purified pink layer in 0.88 M sucrose, a medium that minimizes artifacts due to aggregation and agglutination, and recentrifuging, there was no further separation into the characteristic mitochondria and microsomes.

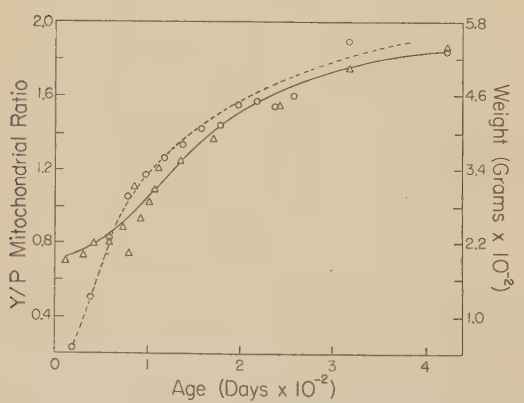

FIG. 1. Correlation of mitochondrial composition of liver cells with age.

The mitochondria–P and the microsomes behave similarly, have the same order of specific radioactivity of cholesterol, which, in turn, differs sharply from that of the mitochondria–Y fractions. This parallelism of the mitochondria–P and the microsomes breaks down in the comparison of the amino acid incorporation of these 2 fractions for the 3 different amino acids tested. This is also true for the radioactivity of the residual non-protein fraction and for the ratios of the nucleic acid to protein radioactivity.

The specific radioactivity of the microsome protein is consistently about twice that of the mitochondria-P and at least 3 times that of the mitochondria-Y. The non-protein radioactivity of the microsomes is significantly higher than that of the mitochondria–P and mitochondria–Y for the 3 amino acids. Finally, the nucleic acid to protein radioactivity ratio is more than twice as great in mitochondria–P than in the microsomes.

In view of the fact that the mitochondria–P exhibits differences in the metabolic properties listed above from the usually accepted mitochondrial fraction and from the microsomes, it may be a distinct type of cytoplasmic particle.

Summary. 1. The cytoplasm of liver has been fractionated by centrifugation in sucrose solution into microsomes, an upper pink mitochondrial layer and a lower normal, yellow mitochondrial layer. These fractions have been studied with respect to amino acid incorporation, cholesterol formation and the ratio of nucleic to protein radioactivity. The mitochondria–P differed sufficiently in these properties from the normal microsomes and mitochondria to be considered a distinct type of

cytoplasmic particle. 2. The curve of the ratios of the lower, normal mitochondria to that of the top layer was found to parallel the weight curve of the rat upon aging. The total amount of mitochondria per unit weight of liver remained fairly constant, with the lower fraction increasing and the upper decreasing with age.

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Metabolism of Intravenously-Infused Sorbitol. (21792)

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Sorbitol administered intravenously to dogs by Todd, *et al.*(1) and rabbits by Seeberg, *et al.*(2) resulted in the development of only moderate levels of sorbitol in the blood, but caused a prompt elevation of blood reducing sugar. Todd, *et al.* attributed this to the formation of glucose from the administered sorbitol. On the other hand, from early liver perfusion experiments, Embden and Greisbach(3) obtained evidence which suggested that fructose rather than glucose was the initial oxidation product of sorbitol. The latter view recently received strong support from Blakley(4) who demonstrated the presence of a sorbitol dehydrogenase in mammalian liver and kidney. However, to our knowledge, it has not been established whether the immediate increase in blood reducing sugar in the intact animal, following intravenous sorbitol, represents conversion to fructose, glucose or possibly some other reducing sugar such as sorbose. Therefore, blood from rabbits undergoing intravenous infusion with sorbitol was studied in the following experiments.

Procedure. Our general procedure for the study of blood and urine during intravenous infusion of carbohydrates in rabbits has been described(2). Sorbitol was administered by continuous intravenous infusion as a 10% solution at the rate of 1 g/kg/hour for 1½ hours. Blood samples were obtained by means of a stationary cannula inserted into the left femoral artery under local anesthesia. Blood fructose was first determined in the samples by the method of Roe, *et al.*(5). A Somogyi(6) titrimetric assay for reducing substances was then made on another portion of the same sample, and the difference expressed as glucose. Osazones of the sugars present were also made and checked for identity. During analysis of the data, a blood glucose rise noted during the first half hour of infusion was suspected of being caused by operative procedures. Therefore, a second series of animals was infused with an equal volume of saline to provide values which could be used to cancel the effect of manipulation.

Results. The mean blood glucose and blood fructose values and their respective standard deviations during 7 sorbitol infusions are shown in Fig. 1. Infusion of sorbitol was accompanied by a prompt fructosemia which reached a mean peak value of 50 mg % at the 1½-hour period, at which time the infusion was stopped. Blood fructose levels were fairly uniform among animals at comparable periods. Levels fell rapidly after cessation of the infusion and only small amounts of fruc-

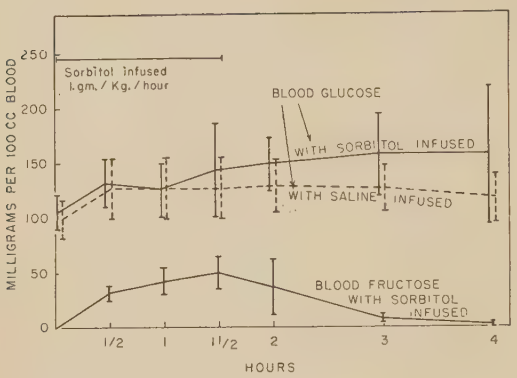

FIG. 1. Blood glucose and blood fructose values during and following intravenous infusion of sorbitol to rabbits.

tose were present 2½ hours later. Blood glucose values did not change during the first hour of infusion. The levels actually went up during the first half hour of infusion, but an almost identical rise was noted in the saline-infused animals, so that no significance can be attributed to this change other than that the experimental procedures employed in restraining the animal and beginning the infusion caused a mean rise of about 20 mg % in blood glucose. No further change is seen in either group between the ½-hour and 1-hour periods of infusion. After the 1-hour interval, an erratic rise in blood glucose occurred. All mean blood glucose values of sorbitol-infused animals are higher after 1-hour interval than those of the saline-infused animals, although because of overlap, none of these means are significantly different except at the 3-hour period ($P = .04$). Thus, a definite, but variable, secondary conversion to glucose can be concluded.

These findings in the intact animal are in direct agreement with Blakley's findings in rat liver slices and homogenates where a primary oxidation of sorbitol to fructose followed by a secondary conversion to glucose was also observed. The identity of the sugars in blood during and following sorbitol infusion was further checked by osazone formation both before and after fermentation of blood samples with yeast and subsequent deproteinization. Filtrates of non-fermented blood yielded only typical glucosazone crystals. Filtrates from blood fermented with yeast did not yield osazones, indicating the absence of sorbose.

Excretion. We have previously determined the average urinary excretion of carbohydrate following sorbitol infusion at the above rate (1 g/kg/hr) to be 17.7% of the amount administered. About three-fourths of the excreted carbohydrate was in the form of unchanged sorbitol and one-fourth as reducing substances calculated as fructose. Subsequently, additional infusions have been made in 4 rabbits at a slower rate (.33 g/kg/hr) to determine whether urinary excretion would thereby be reduced. Method of urine collection and analysis is described in the previous publication. Results are shown in Table I. At the slower rate, it may be noted that excretion was significantly reduced, but not in proportion to the reduction in rate. The excretion of a small amount of sorbitol at this slow rate of administration in the face of rapid metabolism, is undoubtedly due to the reported lack of reabsorption of sorbitol by the renal tubule(7).

Summary. 1. Intravenous infusion of sorbitol in rabbits causes a prompt fructosemia which subsides relatively rapidly following cessation of infusion. A more variable secondary glucosemia occurs following the rise in blood fructose and during the subsidence of

TABLE I. Urinary Excretion of Carbohydrate following Intravenous Infusion of Sorbitol to Rabbits, .33 g/kg/Hr for 4½ Hr, Expressed as % of Sorbitol Administered.

Wt, kg	Total sorbitol administered, g	Sorbitol excreted mg	Sorbitol excreted %	Glucose excreted mg	Glucose excreted %	Fructose excreted mg	Fructose excreted %	Total carbohydrate excreted mg	Total carbohydrate excreted %
4.7	6.44	566	8.8	186	2.9	38	.6	790	12.3
3.9	5.34	325	6.1	11	.2	32	.6	368	6.9
3.9	5.34	373	7.0	16	.3	11	.2	400	7.5
4.4	6.03	904	15.0	42	.7	18	.3	964	16.0

Mean total carbohydrate excreted, 10.2%

the fructosemia. These findings in the intact animal are in accord with previous observations by others on sorbitol metabolism by rat liver slices and homogenates. 2. Although rapidly metabolized, a small amount of sorbitol appears in the urine with infusion rates as low as .33 g/kg/hr.

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Composition of Brain Pentose Nucleic Acid in Normal and Poliomyelitis Virus Infected Mice.* (21793)

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While the composition of nucleic acids of a wide variety of organs and tissues from several animal species has been intensively investigated(1,2), that of the brain has been virtually neglected. Except for a recent brief analysis of the nucleotides of the brain of the cat(3), there have been no reports in the literature on the composition of either the pentose nucleic acid (PNA) or deoxypentose nucleic acid (DNA) of brain tissue. However, to the investigator in the field of neurotropic viruses it could be of some significance to know the composition of brain nucleic acids and how these might fluctuate with central nervous system defection.

The present report is of a study undertaken to analyze the purine and pyrimidine composition of the cytoplasmic pentose nucleic acid of normal and poliomyelitis virus infected mouse brains. The analysis of PNA rather than DNA was selected because of the generally greater abundance and metabolic activity of the former. In addition, earlier radiophosphorus studies on the turnover rate of nucleic acids in the brain had revealed negligible P^{32} incorporation in the DNA fraction, while specific activities with respect to cytoplasmic PNA were readily measurable(4). Since it has been shown in the case of a number of other animal organs, as well as brain, that the renewal rate of deoxyribonucleic acid phosphorus is generally low(5), a similar set of experiments was carried out with a differently tagged molecule. Here, using 4,6-C^{14} adenine, it also was found that a significantly greater incorporation of the labeled purine took place in the cytoplasmic pentose nucleic acid(6).

Methods. The brains of 4 to 5-week-old Webster strain white mice were collected in iced beakers and homogenized in 9 volumes of cold 0.25 M sucrose in a Potter-Elvehjem tube. The homogenate was centrifuged in an International refrigerated centrifuge at 2000 r.p.m. for 10 minutes to sediment nuclei and red blood cells. The supernatant fluid was decanted and the sediment rehomogenized in nine volumes of cold 0.25 M sucrose. The resulting supernatants were pooled and the cytoplasmic ribonucleic acid isolated by the method of Payne(7) modified from Barnum (8). The fluid was made 5% to trichloroacetic acid (TCA) to precipitate protein and ribonucleoprotein, and after centrifugation and decantation was washed with cold 95% methyl alcohol followed by 3 extractions in

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chloroform-methyl alcohol (1:3) and a final methyl alcohol extraction. To split off the protein, the lipid-free precipitate was mixed with ice-cold 5% NaOH and left at 0°C for 2 hours. Glacial acetic acid was added to pH 6.5 to precipitate the protein, the precipitate was filtered through tissue paper and the sediment discarded. Pentose nucleic acid was precipitated by bringing the solution to pH 2 by the addition of 5 N HCl, adding an equal volume of 95% methyl alcohol, and then storing at 0°C for 1½ hours and centrifuging in the cold. The PNA was purified by dissolving in 0.05 M $NaHCO_3$ and reprecipitating with HCl and methyl alcohol, until there was no longer material insoluble in 0.05 M bicarbonate. It was then washed with methyl alcohol followed by a wash in anhydrous ether, and allowed to dry. The isolation of pentose nucleic acid was carried out similarly with normal and poliomyelitis infected mouse brain material. Mice were inoculated intracerebrally with 0.03 ml of a suspension of MEF_1 strain (Type 2) poliomyelitis virus containing approximately 50 LD_{50}. The infected brains were harvested periodically from animals showing flaccid paralysis, worked through the first methanol wash and then stored in the cold. When a total of 40 brains had been brought to this step, the procedure was continued as usual to completion. The brains of 44 uninoculated mice were harvested for the analysis of normal tissue. The analysis of PNA was performed by 2 different methods. The first was essentially that of Marshak and Vogel(9), namely, hydrolysis with 72% perchloric acid and chromatography in an aqueous HCl-isopropanol solvent. The second method was that of Smith and Markham(10). This latter method was used additionally because of a report by Wyatt (11) that the presence of protein could interfere with the cleavage of pyrimidine ribosides by perchloric acid. In the method of Smith and Markham hydrolysis was carried out with 1 N HCl and the hydrolysate was chromatographed in a tertiary butyl alcohol-HCl solvent. The quantities of the purine and pyrimidine bases in the eluates of the spots were calculated on the basis of the optical densities at the absorption maxima. In the case of $HClO_4$ hydrolysis the molar extinction coefficients as given by Wyatt(11) were used, while with HCl hydrolysis the molar extinction coefficients as given by Knight(12) were employed.

TABLE I. Purine and Pyrimidine Composition of Brain Pentose Nucleic Acid from Normal and Poliomyelitis Virus Infected Mice.

Constituent	Per 4 moles		Ratio to adenine 1.00	
	Normal	Infected	Normal	Infected
	A. $HClO_4$ hydrolysis			
Adenine	.88±.01*	.83±.01	1.00	1.00
Guanine	1.34±.01	1.25±.01	1.53±.03	1.49±.02
Cytosine	1.24±.02	1.21±.01	1.43±.01	1.45±.02
Uracil	.54±.03	.71±.01	.63±.04	.85±.00
	B. HCl hydrolysis			
Adenine	.90±.01	.88±.01	1.00	1.00
Guanine	1.34±.02	1.31±.02	1.49±.03	1.49±.01
Cytosine	1.21±.02	1.19±.02	1.34±.01	1.35±.02
Uracil	.55±.01	.62±.02	.61±.01	.71±.03

* ± stand. error of mean $= \sqrt{\Sigma d^2/n(n-1)}$.

Results. - The method of isolation described did not give completely pure PNA preparations. However, the extent of contamination from preparation to preparation, both from normal and infected brain, was quite consistent. The DNA contamination as determined by the method of Ceriotti(13) ranged between 1.0 and 1.4%, while protein as measured by the Lowry test(14) was present to the extent of about 13% of the PNA by weight. The method of preparation and the analytical methods employed were highly reproducible. The analysis of different normal brain preparations by both hydrolytic and chromatographic procedures gave values for the individual purine and pyrimidine bases which generally agreed well within 3%. Evidently the amounts of protein in the preparations were not large enough to interfere with the perchloric acid hydrolysis, and this procedure thus could be considered adequate and reliable for comparative quantitative determinations.

In Table I are given the results obtained in the analysis of the PNA base composition of normal and poliomyelitis virus infected mouse brains. Sections A and B represent the findings with the two methods of hydrolysis employed. The values shown are the means of

triplicate samples and the standard errors of the means.

A comparison of the composition of normal and infected brain PNA reveals the main difference to be in the uracil content, which appears higher in infected than in normal brain PNA. Calculation of the base composition per 4 moles reveals an increase in uracil alone with a concomitant decrease in the content of the other bases. If, instead, the composition is computed taking adenine arbitrarily as 1.00, the only marked difference noted is in uracil. The same holds true if ratios are calculated taking guanine or cytosine arbitrarily as 1, whereas if uracil is taken as 1 all the other bases in infected brain PNA show a marked drop as compared with those of normal brain. The increase in uracil content, then, seems to be the most important difference in mouse brain PNA as a consequence of infection with poliomyelitis virus.

Since the differences observed were not very great, it was considered necessary to determine their significance statistically. The "t" test employed for this purpose indicated that the probability of getting the observed difference by chance was less than 1% in the case of $HClO_4$ hydrolysis and slightly more than 3% in the case of HCl hydrolysis; while the "t" test for the combined data of the two procedures employed was at a significance level of less than 0.1%. From statistical considerations, then, the differences in uracil, even if small, appear significant.

The implications of these findings for nucleic acid metabolism in poliomyelitis are difficult to assess. They may indicate an enhanced propensity of brain cells in the infected animal to synthesize PNA of a new type. Or, perhaps, in the process of cellular degeneration a molecular species or residue of pentose nucleic acid richer in uracil is broken down less rapidly than other molecular species in the cell. Also, the results represented here must be considered minimal, since many of the cells of the brain are not infected with virus(15). It could well be, then, that a PNA analysis of only cells which were affected would reveal a much more pronounced change in uracil content over that of brain cells from normal mice. Hence this change might represent a truly major compositional change, and may reflect a significant alteration in nucleic acid metabolism with viral infection.

In view of the dependence of the composition of pentose nucleic acids upon the methods of preparation(16,17) two other methods have been applied. In one method the lipid-free nucleoprotein preparation was suspended in 15 N NH_4OH for 24 hours at 37°C, and the DNA was then precipitated with 5% TCA. Following decomposition of the TCA by heating, the PNA supernatant was hydrolyzed and chromatographed by the Smith-Markham method. The resulting chromatograms were usually not satisfactorily clean and the analyses were not very reproducible. In another procedure, extracting the PNA by heating at 95°C in 5% TCA(18) gave reproducible preparations but resulted in a reduction of almost 40% in the cytosine component. This apparently is not unique for brain tissue, as it may be observed also in the case of mouse liver where extraction with hot TCA elicited a marked drop in the cytosine analysis compared to extraction with guanidine hydrochloride(19,20).

A comparison with the findings for PNA composition of mouse liver(19) and cat brain (3) reveals the PNA in each instance to contain more guanine than adenine and more cytosine than uracil. Also, it is noted that cat brain PNA is richer in uracil than either mouse brain or mouse liver PNA, while the latter contains more guanine than the brain PNA of the cat or mouse. It is difficult to conclude, as has been suggested by Chargaff (2), that mouse brain PNA resembles more nearly cat brain (an homologous organ of a different species) PNA than mouse liver (an heterologous organ of the same species) PNA; this because the methods employed for the isolation, purification and analyses of the bases were quite different in each case.

Summary. The purine and pyrimidine composition of brain cytoplasmic pentose nucleic acid from normal mice and mice infected with the MEF_1 strain of poliomyelitis virus was investigated. For this purpose, two different methods of hydrolysis and chromatography were applied to isolated and purified

PNA preparations, and the results were found to be in good agreement. A small but apparently significant rise in uracil content in pentose nucleic acid was observed in the brains of mice showing paralytic symptoms of central nervous system involvement.

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Primary Isolation of Influenza A, B, and C Viruses in Monkey Kidney Tissue Cultures.* (21794)

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The usual laboratory procedure for primary isolation of influenza viruses from throat washings or swabs has been inoculation of embryonated hen's eggs. Less frequently, ferrets, mice, and hamsters have been used. Recently a new method employing human embryonic and monkey kidney tissue cultures for isolating influenza A and B was described by Mogabgab *et al.*(1-3).

From throat samples collected during epidemiological studies of respiratory illnesses among familial and general population groups in the Washington, D.C. metropolitan area during early 1955, a number of agents cytopathogenic for cultures of monkey kidney cells were isolated. These agents in tissue culture fluids were found to agglutinate both chicken and guinea pig erythrocytes. In subsequent passage in the amniotic sac of 11-day-old chick embryos the agents were established as shown by the presence of hemagglutinins at high titers. Hemagglutination-inhibition tests identified these strains as influenza B. All strains were similar and resembled closely the B/GL/1/54 (GL-1760-54B) strain isolated at the Great Lakes Naval Training Center in Illinois(4). Although a few strains of influenza B were isolated in embryonated eggs, early observations indicated that isolations of virus were more readily and easily obtained through the use of monkey kidney tissue cultures than embryonated eggs. The studies reported here were therefore undertaken in order to evaluate this method for primary isolation

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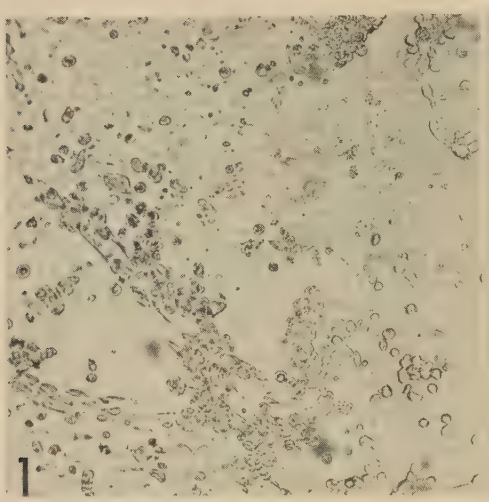
of influenza viruses.

Materials and methods. Samples. Throat swabs were taken from each individual studied, combined with garglings when obtained, and placed in screw-cap vials containing 10 ml sterile beef heart bacteriological broth. These were placed in a CO_2 ice box within 2-3 hours and kept frozen until used. Throat specimens from the 1953 influenza A epidemic contained skim milk as diluent and virus had previously been isolated from about half of the samples used in these experiments. These samples had been stored for approximately 2 years in a CO_2 ice box. *Preparation of monkey kidney tissue cultures.* Stationary cultures of monkey kidney cells were prepared according to Youngner's technic(5). The initial growth medium (purchased from the Microbiological Associates, Bethesda, Md.) consisted of 97.5% Hank's solution, 0.5% lactalbumin, and 2% calf serum. Prior to inoculation of cultures, the medium was changed to 75% Earle's solution, 24% ox serum ultrafiltrate, and 1% inactivated horse serum, and contained 250 units penicillin and 250 μg streptomycin/ml. *Virus isolation in monkey kidney tissue cultures.* Samples were inoculated into tissue cultures and embryonated eggs at the same time to eliminate the effects of repeated freezing and thawing on the sample. Two tubes of monkey kidney cultures were inoculated from each sample, each tube receiving 0.9 ml of culture medium and 0.1 ml of the broth sample. Later 0.2 ml of broth inoculum was found non-toxic and is now used routinely. Tissue cultures were examined daily for cytopathogenic effects and the fluid medium changed every third or fourth day. Fluids from tubes showing typical cytopathogenic changes were tested by hemagglutination using 0.4% suspension of washed chicken and sometimes guinea pig erythrocytes. In the early phases of this study, fluids which contained hemagglutinins were immediately inoculated intra-amniotically into 11-day-old embryos. Amniotic and allantoic fluids were separately harvested after 48 hours incubation and tested for hemagglutinins. These were usually of sufficiently high titer after one egg passage to permit typing by the hemagglutination-inhibition test, using rooster antiserum prepared against the following strains: A/PR8, A/FLW/1/52, B/Lee, B/Va/1/50, B/GL/1/54, and C/1233. Occasionally one or 2 egg passages were necessary, but all influenza strains isolated in tissue culture were adapted to chick embryos. It was later discovered since hemagglutination titers of 1/40 or higher could be obtained in tissue culture fluids of either first or second passages, that it was possible to type strains directly from tissue culture fluids. This eliminated egg passages and shortened the time required for identification. *Virus isolation in chick embryos.* For each sample, 6 11-day-old embryos were inoculated intra-amniotically with 0.2 ml and incubated 5 days at 36°C. Living embryos were chilled overnight at 4°C and lungs and amniotic fluids were harvested. The lungs were ground in alundum using the amniotic fluid as diluent and the suspension inoculated into another group of 6 eggs intra-amniotically. After 48 hours incubation, living embryos were chilled and amniotic and allantoic fluids were harvested separately and tested for hemagglutinins. Negative fluids were carried through a third amniotic passage.

Results. 1. *Isolations from 1955 influenza B epidemic.* Of the 78 samples obtained during the 1955 epidemic in the Washington area and tested in both chick embryos and monkey kidney tissue cultures, one isolation of influenza B (1.3%) was obtained in chick embryos compared to 17 isolations of influenza B (21.8%) and one of influenza C (1.3%) in monkey kidney cultures. Virus was isolated in monkey kidney cultures from the one sample from which influenza B was obtained in chick embryos. The results of this experiment showed that influenza B virus was more readily isolated in monkey kidney tissue cultures than in chick embryos. Cytopathogenic effects were usually first observed between 3 to 7 days after inoculation, but 2 strains caused cytopathogenic changes 9 and 11 days later. The type of cytopathogenicity seen was the same as that described by Mogabgab *et al.*(3). The most striking effect was a rounding of the cells often followed by the appearance of small masses of clumped, rounded cells (Fig. 1 and 2), which rapidly involved most of the cells in the cultures. Hemagglutination tests



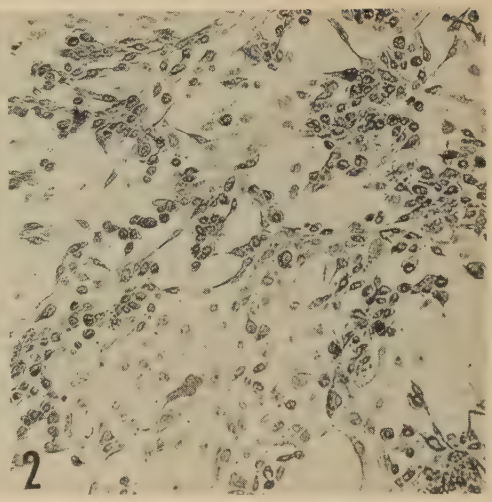


FIG. 1. Cytopathogenic effects of influenza B in monkey kidney tissue culture. Unstained (×87).

FIG. 2. Cytopathogenic effects of influenza B in monkey kidney tissue culture. H and E stain (×87).

on fluids taken at the time of maximal effects showed that cytopathogenic effects were always associated with hemagglutinin production; on the other hand, neither hemagglutinins nor virus was ever recovered from normal appearing cultures. Thus, fluids from 35 sets of cultures that showed no evidences of cytopathogenic effects were negative in the hemagglutination test. Attempts to isolate virus from these fluids both by second tissue culture passages and egg inoculations were uniformly unsuccessful. It therefore appears that these strains of influenza B do not multiply in monkey kidney cells without destruction of the affected cells.

Two strains of Type 3 adenoidal-pharyngeal-conjunctival viruses were isolated in monkey kidney cultures and each was found in a sample which also contained influenza viruses, one with influenza C and the other with influenza B. In both instances, although cytopathogenic effects were observed, the tissue culture fluids were negative for hemagglutinins. These fluids were passed simultaneously into HeLa cell cultures and chick embryos. Cytopathogenic changes characteristic of those produced by the APC viruses were noted in the HeLa cultures after 3 to 4 days incubation. Neutralization tests performed according to the technic described by Rowe *et al.*(6) established the identification of the the viruses as Type 3 APC. In chick embryo passages of the tissue culture fluids, the influenza C strain was detected in the fourth amniotic passage while the influenza B strain required 2 amniotic passages. In these 2 instances where 2 different viruses multiplied within the same tissue cultures, separation and identification of the viruses was readily obtained due to the difference in growth in other host tissues.

2. *Influenza A isolations.* A similar experiment using 40 samples taken in the 1953 epidemic of influenza A was conducted in an attempt to evaluate the 2 methods of isolation for influenza A virus. These samples had been taken in skim milk, however, and since this diluent proved to be deleterious to monkey kidney cultures second tissue culture passages were found necessary. Eleven isolations of influenza A virus were made in monkey kidney tissue cultures from the 40 selected samples tested and 18 isolations of influenza A were made in chick embryos. It was not possible by this experiment to determine whether monkey kidney cultures are a better means for isolating influenza A virus than chick embryos, because of the unfavorable effect of skim milk on tissue cultures. We have confirmed the observation by Mogabgab *et al.*(1,2) that in-

fluenza A virus from primary isolation in tissue culture did not agglutinate chicken erythrocytes. Guinea pig erythrocytes were agglutinated in titers ranging from 1/10 to 1/80, however. After 5 serial tissue culture passages of one strain, the fluids were found to have a titer of 1/10 with chicken erythrocytes, and 1/80 with guinea pig erythrocytes. After one intra-amniotic passage in chick embryos, all strains of influenza A isolated in tissue cultures agglutinated chicken erythrocytes in titers of 1/80 or higher.

3. *Influenza C isolations.* Although comparative studies on the isolation of influenza C in monkey kidney cells and chick embryos were not made, we isolated one strain of influenza C virus in monkey kidney tissue cultures from the 78 samples collected in 1955. Three other strains of influenza C were isolated in monkey kidney tissue cultures from selected samples obtained from the Great Lakes Naval Training Center.† Hemagglutination titers of tissue culture fluids, using chicken red blood cells and tested at 4°C were high enough (1/40 to 1/160) to permit direct typing of these strains. The cytopathogenic effects produced in monkey kidney cultures by influenza C were similar to those produced by influenza A and B.

Summary. 1. Influenza virus isolations were attempted simultaneously in monkey kidney tissue cultures and 11-day embryonated eggs from 78 throat samples taken during the 1955 epidemic in the Washington, D.C. area. A higher percentage of influenza B isolations was made in monkey kidney tissue cultures (21.8%) than in embryonated eggs (1.3%). Influenza C and Type 3 APC viruses were also recovered in the monkey kidney tissue cultures from some of these samples. Influenza A virus and influenza C virus were also isolated in monkey kidney cultures from other samples, but whether this method offers a better medium than the usual embryonated eggs was not determined for these types. 2. Cytopathogenic changes in monkey kidney cultures produced by influenza A, B, and C viruses appeared to be similar. These changes were readily recognized, and were associated with the production of hemagglutinins. 3. The use of monkey kidney cultures offered distinct advantages over chick embryos: (a) current strains of influenza B virus could be more readily isolated, (b) the time required for isolation and identification of the virus was shortened and (c) other respiratory tract viruses such as the Type 3 APC virus could be isolated.

† Kindly supplied by Cmdr. John R. Seal (MC), USN.

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Protective Effect of S,β-Aminoethylisothiuronium•Br•HBr and Related Compounds Against X-Radiation Death in Mice.* (21795)

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The ability of cysteine, BAL, thiourea, glutathione, and β-mercaptoethylamine (MEA) to modify the X-ray sensitivity of mice is generally ascribed to the sulfhydryl or potential sulfhydryl groups(1). In simpler systems, such as bacterial suspensions, cysteine and BAL protect in part by a reduction in the oxygen tension of the medium surrounding the cells(2). Other processes are probably involved in MEA, which appears to be unique in its effect on the radiosensitivity of *Escherichia coli*(3). The striking "dose-reduction" factors obtained when this compound is added to the bacterial suspensions have led to a systematic modification of the basic structure of MEA in an effort to define the limiting configuration necessary for protective activity in compounds of the type $R(CH_2)_xSR'$. Initial experiments have shown that certain alterations can be made without the loss of its protective activity in mice; a preliminary report therefore seems warranted at this time. Studies with bacterial suspensions are to be presented elsewhere.

Methods. Males from 2 inbred strains of mice, C_{57}BL 8-10 weeks old and C_3H 10-12 weeks old, and a hybrid strain 101 X C_3HF_1 12-14 weeks old, were used in this investigation. Fresh solutions of the test compound in volumes of 0.2-0.4 ml, adjusted to pH 6.5-6.8, were given to the mice by intraperitoneal injection 10 minutes prior to total-body X-irradiation. The dosage of the compounds was, in all cases, insufficient to cause any deaths. The handling and irradiation procedures have been previously described(4).

Results. The protective effectiveness of several of the compounds tested with 101 X C_3H and C_3H mice is shown in Table I as the percentage survival at 28 days after X irradiation. In Table II, similar data are presented for C_{57}BL mice as well as the mean survival time for those cases with no 28-day survivors. The dosage is given in micromoles per mouse as well as in mg/kg of body weight to facilitate the comparison on a molecular weight basis of the protective activities of compounds of similar structure. Two new compounds, S, β-aminoethylisothiuronium.Br. HBr (AET) and S, γ-aminopropylisothiuronium • Br • HBr (APT) provide effective protection against X radiation (800 r). In another series of experiments, the 28-day LD_{50} for C_{57}BL and 101 X C_3H male mice treated with either AET or MEA was found to be about 900 and 1150 r, respectively. However, the results in Table I show that AET is more effective than MEA on an equimolar basis since 18 μM of AET gave almost as much protection to the 101 X C_3H mice, as indicated by the 28-day survivals, as 44 μM of MEA. Also with C_3H mice, 17 μM of AET was more effective than 34 μM of MEA, and 17 μM of MEA was completely ineffective. In this C_3H strain, 16 μM of the APT was somewhat more effective than 17 μM of AET.

Since male mice were not available for the toxicity tests, C_3H females weighing 19-22 g and 10-12 weeks old were used for a comparison of the relative acute toxicity of the three protective compounds as manifested by convulsions and death. The results of the tests are presented in Table III. Comparison, in C_3H mice, of the effective dose for protection against radiation injury with the acute toxic dose shows that MEA and APT, in contrast to AET, required near-toxic amounts to increase the 28-day survival of mice given 800 r of X-rays.

In contrast to the protective compounds with a guanyl or hydrogen as R', conversion to the disulfide resulted in a loss of activity. In every case tested, modification of the amino group of MEA or AET by acylation, alkyla-

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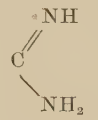
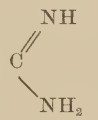
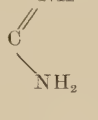
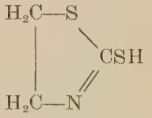
TABLE I. Effect of S,β-Aminoethylisothiuronium · Br · HBr and Related Compounds on Survival of C_3H and 101 X C_3H Male Mice Exposed to 800 r of Total-Body X-Radiation.

Compound	Structure R	Structure $(CH_2)_xS$	Structure R′	Dose μM/mouse	Dose mg/kg	Strain	28-day survival* (%)
Saline controls	—	—	—	—	—	101 X C_3H	6
	—	—	—	—	—	C_3H	0
S,β-Aminoethylisothiuronium · Br · HBr (AET)	NH_2	X = 2		18	250	101 X C_3H	88
				34	480	C_3H	80
				17	240	C_3H	33
S,γ-Aminopropylisothiuronium · Br · HBr (APT)	NH_2	X = 3		16	240	C_3H	60
				8	120	C_3H	13
β-Mercaptoethylamine · HCl (MEA)	NH_2	X = 2	H	44	250	101 X C_3H	100
				34	180	C_3H	7
				17	90	C_3H	0
S,S-Methyldiisothiuronium dibromide		X = 1		15	245	101 X C_3H	13
S,S-Ethyldiisothiuronium "		X = 2		15	255	101 X C_3H	0
S,S-Propyldisothiuronium "		X = 3		14	250	101 X C_3H	0
2-Mercaptothiazoline				26	100	101 X C_3H	0
N,Acetyl-β-mercaptoethylamine	CH_3CONH	X = 2	H	42	250	101 X C_3H	17
N,S-Diacetyl-β-mercaptoethylamine	CH_3CONH	X = 2		35	280	101 X C_3H	25

* Three trials of 6 mice each for a total of 18 101 X C_3H mice. Three trials of 5 mice each for a total of 15 C_3H mice.

TABLE II. Effect of S,β-Aminoethylisothiuronium • Br • HBr and Related Compounds on Survival of C_{57}BL Male Mice Exposed to Total-Body X-Radiation.

Compound	Dose μM/mouse	Dose mg/kg	X-ray dose (r)	28-day survival (%)*
Saline controls	—	—	950	0
S,β-Aminoethylisothiuronium • Br • HBr (AET)	32	450	950	70
β-Mercaptoethylamine • HCl (MEA)	44	250	950	56
				Mean survival time (days)†
Saline controls	—	—	800	4.9
bis(β-Aminoethyl) disulfide	34	280	800	9.4‡
N-Acetyl-β-mercaptoethylamine	42	250	800	8.2‡
N,S,Diacetyl-β-mercaptoethylamine	35	280	800	9.6‡
β-Dimethylaminoethylisothiuronium • Br • HBr	23	350	800	7.6‡

* Two trials of 10 mice each for a total of 20 mice.
† No 28-day survivors.
‡ Significantly different from saline controls at the 1% levels.

TABLE III. Acute Toxicity of 3 Protective Compounds in C_3H Female Mice.

Compound	LD_5 μM/mouse	LD_5 mg/kg	LD_{50} μM/mouse	LD_{50} mg/kg	LD_{95} μM/mouse	LD_{95} mg/kg
β-Mercaptoethylamine (MEA)	39	225	48	275	56	320
S,β-Aminoethylisothiuronium • Br • HBr (AET)	44	620	49	690	53	750
S,γ-Aminopropylisothiuronium • Br • HBr (APT)	19	280	23	340	27	400

tion, or replacement with a different group lowered or erased protective activity (Tables I, II), although several of the compounds significantly increased the survival time. Other derivatives which gave little or no protective activity, but not shown in the tables, include β-cyano- and β-carboxyethylisothiuronium • Br.

Conclusions. (a) Replacement of the sulfhydryl group of β-mercaptoethylamine with the isothiuronium group yields a compound (AET) with increased protective activity and greater chemical stability. (b) A free amino group beta or gamma to the sulfhydryl or isothiuronium group is necessary for protective activity. (c) In the aminoisothiuronium series, the carbon chain can be lengthened by one carbon atom without the loss of protective activity. (d) S,β-aminoethylisothiuronium • Br • HBr provides effective protection against radiation injury at dose levels considerably less than the dose required to produce acute toxic effects.

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Improved Substrate for Study of Clearing Factor. (21796)

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Hahn(1) observed that heparin injected into dogs cleared the lipemic serum. This discovery stimulated a great deal of interest in the fields of lipid and lipoprotein metabolism. Studies on lipemia clearing can be facilitated by a readily available stable substrate whose rate of reaction can be expressed linearly. Previous reports have dealt with the clearing of lipemic plasma(2-6), serum lipoproteins(7), egg lipoproteins(8), chyle (9), homogenized milk(10), and oil emulsions (5,7,11-13).

We have found that a commercially prepared 50% coconut oil emulsion (Ediol, Schenley Laboratories, Inc.) mixed with the proper amounts of normal plasma or albumin becomes a suitable substrate for a rapid quantitative test.

Materials and methods. Ediol was diluted 1:100 with physiological saline, resulting in a 0.5% oil emulsion. Normal dog blood was collected in 1/10 volume of 3.2% trisodium citrate after 18 hours of fasting and the plasma was separated by centrifuging at 2000 r.p.m. for 15 minutes. The plasma was used the same day or frozen until needed. Heparinized dog blood was prepared by the intravenous injection of 50 μ heparin per kg body weight of dog after it had been fasting for 18 hours. The blood was collected 10 minutes later in 1/10 volume of 3.2% trisodium citrate. The plasma was separated, used the same day or frozen until needed. Bovine albumin (Bovine Albumin, 22% Solution, For Rh Testing, Ortho Pharmaceutical Corp.) was diluted 1 ml to 7 ml, or approximately to 3% albumin with 0.32% trisodium citrate. Phosphate buffer of pH 7.2, ionic strength 0.1 diluted with an equal volume of physiological saline was used to adjust the volumes in the tubes being tested. For a 1 ml test system, 0.1 ml of the 0.5% oil emulsion plus 0.5 ml of 3% albumin or 0.5 ml of normal plasma was pipetted into a Lumetron micro tube, 50 mm x 9.5 mm O.D. One of these mixtures, the heparinized plasma, and the phosphate saline buffer were heated in separate tubes in a 37.5°C water bath for 5 minutes. Then 0.4 ml of the heparinized plasma or heparinized plasma diluted with the buffer was pipetted into the micro tube containing the substrate and mixed. Turbidity readings were made immediately and at 5 or 10 minute intervals. The tubes were kept in a 37.5°C bath at all other times. A Lumetron Photoelectric Colorimeter (Photovolt Corp.) with a 660 mμ filter and a micro tube adapter was used. Distilled water was employed as the blank. Results are expressed as increases in per cent light transmission.

Results. Fig. 1 shows the increase in light transmission of the oil emulsion substrate when acted upon by heparinized plasma. In 2 ml test systems, there was a decrease in turbidity which varied with the amount of heparinized plasma between the limits of 5% and 40% heparinized plasma. Normal plasma was ineffective in all the runs made. The curved lines in Fig. 1 may lead to errors in assay. It would be desirable to have a straight line curve illustrating the reaction. It has been reported that the addition of normal plasma will increase the *in vitro* clearing reaction(9). We have found that by in-

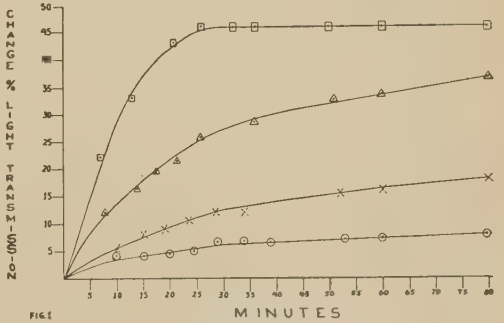

FIG. 1. Increase in % light transmission of oil emulsion as a function of concentration of heparinized plasma: (○) 0.1 ml (5%), (×) 0.2 ml (10%), (△) 0.4 ml (20%), (□) 0.8 ml (40%) heparinized plasma was added to 0.2 ml oil emulsion and the total volumes brought up to 2 ml with phosphate buffer. For the control, 0.4 ml of normal plasma was used.

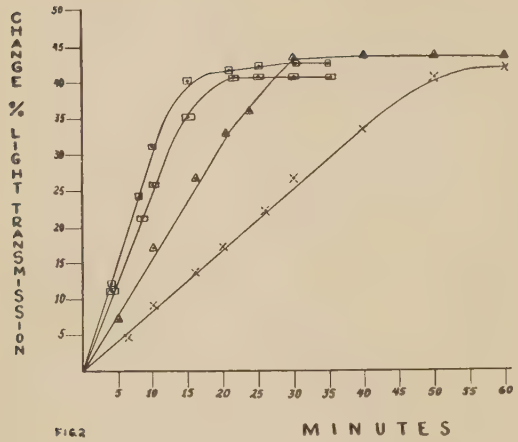

FIG. 2. Increase in % light transmission of oil emulsion and normal plasma as a function of concentration of heparinized plasma: (×) 0.2 ml (10%), (△) 0.4 ml (20%), (▭) 0.6 ml (30%), (□) 0.8 ml (40%), heparinized plasma was added to 0.2 ml oil emulsion and 1 ml normal plasma. Total volumes were brought up to 2 ml with phosphate buffer. For the control, 0.2 ml normal plasma was used.

creasing the concentration of normal plasma to 50% in the oil emulsion substrate, a straight line curve results. The decrease in turbidity was proportional to the concentration of the heparinized plasma in the range from 0.5% to 40%, (Fig. 2 and 3).

The effects of albumin on clearing *in vitro* have been noted in previous studies(14-16). On the addition of albumin in a concentration of 1.5% (which is about equal to the concentration of albumin in the normal plasma added above), straight lines for the reaction were

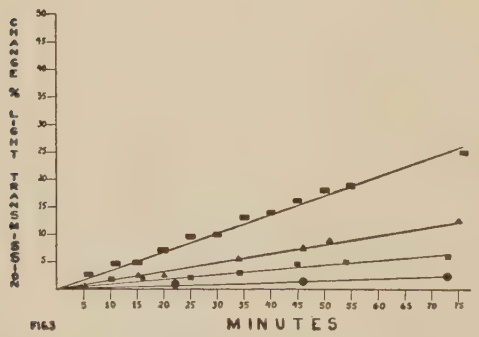

FIG. 3. (●) 0.02 ml (0.5%), (■) 0.04 ml (1%), (▲) 0.1 ml (2.5%), (▬) 0.2 ml (5%) of a 25% solution of heparinized plasma was added to 0.1 ml oil emulsion and 0.5 ml normal plasma. Total volumes were brought up to 1 ml with phosphate buffer. For the control, 0.4 ml of normal plasma was used.

again obtained. Albumin in less than 1% concentrations will not result in straight line reactions. Tests were run (Fig. 4) in 1 ml systems with albumin instead of normal plasma and with heparinized plasma in similar concentrations as shown in Fig. 2.

Fig. 5 shows that the increased change in per cent light transmission was a function of the greater concentration of clearing factor rather than any other serum factors. Normal plasma was added to heparinized plasma and substrate so that total plasma concentration was the same in each tube. The illustrated reactions are of 1 ml systems containing

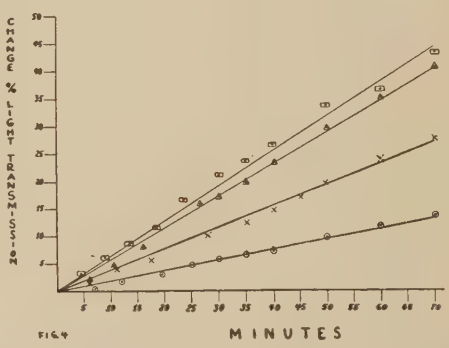

FIG. 4. Increase in % light transmission of oil emulsion and albumin as a function of concentration of heparinized plasma: (○) 0.05 ml (5%), (×) 0.1 ml (10%), (△) 0.2 ml (20%), (▭) 0.3 ml (30%) heparinized plasma was added to 0.1 ml oil emulsion and 0.5 ml 3% albumin. Total volumes were brought up to 1 ml with phosphate buffer. For the control, 0.4 ml normal plasma was used.

0.05% oil and 1.5% albumin, with varying concentrations of heparinized plasma from 5% to 40% and a total plasma concentration of 40% in each tube. The greater the concentration of heparinized plasma, the greater the increase in per cent light transmission. Note that the curves are not straight lines, probably because of the very high protein or albumin concentration.

Discussion. An improved substrate for the assay of clearing factor has been presented. The advantages of this substrate over others is that it is readily obtainable, reproducible, stable, and the clearing reaction can be expressed linearly. Lipemic plasma and chyle are not very stable nor reproducible. Egg lipoprotein, plasma lipoprotein, and homogenized milk clear slowly. The oil emulsion

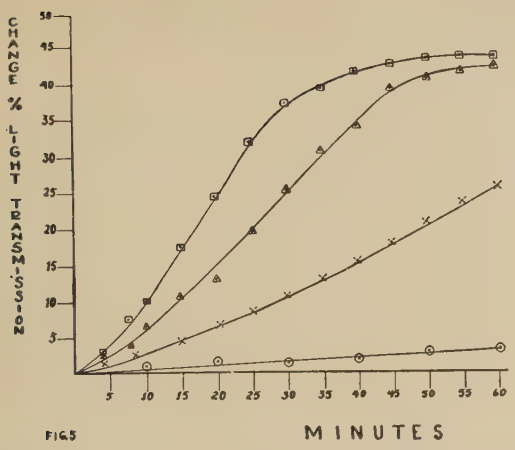

FIG. 5. Increase in % light transmission of oil emulsion and albumin with constant total plasma as a function of concentration of heparinized plasma: (○) 0.05 ml (5%), (×) 0.1 ml (10%), (△) 0.2 ml (20%), (□) 0.4 ml (40%) heparinized plasma was added to 0.1 ml oil emulsion and 0.5 ml 3% albumin. Total volumes were brought up to 1 ml with normal plasma. For the control, 0.4 ml normal plasma was used.

substrates used previously were stable and reproducible but the clearing reaction graph was a curved line. With the substrate described, the results can be read directly from the graph without the calculations that are necessary when using the procedure of Grossman(12).

The diluted Ediol mixed with normal plasma can be a substrate for the assay of purified clearing factor because the cofactors are present in the normal plasma.

Since the graphical representation of the experimental results appears to be dependent, to some extent, on the nature of the substrate, no inference as to the mechanism can be drawn. The linearity of the results with Ediol plotted against change in per cent light transmission may be due to fortuitous circumstances. The data obtained with oil emulsions versus optical density(5,7,11-13), usually were not straight lines.

Addition of normal plasma in 50% concentration or bovine albumin in 1.5% concentration to a test system resulted in straight line curves for the reaction. However, the action of these components was not the same. Addition of plasma to the system increased the change in per cent light transmission quite markedly from zero time to the end. Albumin did not increase the per cent light transmission at the beginning but only after approximately 30 minutes of incubation. Since the concentration of albumin was essentially the same in both cases, it would indicate that albumin is not the only cofactor necessary for the clearing reaction. This conclusion is in agreement with Gordon and coworkers(15), and opposed to Nikkilä and Haahti(16), who claim that electrophoretically prepared albumin did not need a second cofactor.

Summary. A commercially available 50% coconut oil emulsion (Ediol) diluted one hundred fold with physiological saline and mixed with 5 volumes of normal plasma or of 3% albumin is a good substrate for the study of clearing factor in heparinized plasma. Albumin is only one of the cofactors for the clearing factor.

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Comparison of Vitamin B_{12} and Desdimethyl B_{12} Activity in the Chick. (21797)

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Fantes and O'Callaghan(1,2) have described a new analogue of vit. B_{12} in which benzimidazole replaces the 5,6-dimethylbenzimidazole moiety of vit. B_{12}. The new compound, called desdimethyl B_{12}(3), was isolated from fermentation products of *Streptomyces griseus* grown in the presence of *o*-phenylenediamine(1,2). It was about twice as active as vit. B_{12} for an *Escherichia coli* mutant (plate assay) and for *Lactobacillus leichmannii,* organisms commonly used for vit. B_{12} assays. Activity with other microorganisms was not tested. Moreover, desdimethyl B_{12} was reported by Witts and Callendar (personal communication to Fantes and O'Callaghan(1,2)) to be "fully active in three pernicious anemia patients." The lowest level tried was a single dose of 10 γ, though lower amounts of vit. B_{12} are known to give a hematological response. Desdimethyl B_{12} is thought to be identical with, or similar to, a benzimidazole analogue of vit. B_{12} isolated independently by Ford, Holdsworth, and Kon(4,5). Their compound was partially active for *E. coli* (tube method) and was 36% as active as vit. B_{12} for *Ochromonas malhamensis,* a protozoan that has a highly specific requirement for vit. B_{12}. The compound of Ford *et al.* (4,5) was obtained by culturing *E. coli* 113-3 in a medium containing benzimidazole and Factor B (vit. B_{12} without the 5,6-dimethylbenzimidazole nucleotide).

Because of the reported high microbiological activity of desdimethyl B_{12} and because of its reported activity in the treatment of pernicious anemia, it was of interest to compare the activity of this compound with that of vit. B_{12} for growth of the chick.

Methods. Day-old New Hampshire chicks, weighing 35-40 g, were distributed equally in groups of 6 chicks each and raised on wire mesh floors. Feed and water were given *ad libitum.* The experiments were conducted for 4-week periods. The basal vit. B_{12}-low, corn-soybean meal diet, $C17B_{12}$,* contained 20% lard, which has been shown to increase the vit. B_{12} requirement of the chick(6,7). Crystalline vit. B_{12}† was used as the standard. Desdimethyl B_{12} was obtained through the courtesy of Drs. K. H. Fantes and Cynthia H. O'Callaghan.‡ This sample was 86% pure. It contained 2.1 to 2.7% of vit. B_{12}, the remainder of the impurity consisting chiefly of moisture(3). Each compound was dissolved in a small amount of 95% ethyl alcohol, aliquots of which were added to the basal diet.

Results. The 4-week weights obtained with vit. B_{12} and desdimethyl B_{12} are given in Table I. From these weights the values in the last 2 columns were calculated. Desdimethyl B_{12} in the diet has only about 9% (5 to 16%) of the activity of vit. B_{12} for supporting the growth of the chick. When desdimethyl B_{12} was injected, the activity was not significantly different from orally administered desdimethyl B_{12}. Larger amounts were not used because of the limited availability of the compound. Whether or not the small activity of the preparation is due to impurities (other than vit. B_{12}, for which an adjustment was made) or to desdimethyl B_{12} itself must wait further experimentation. In spite of the reported high activity of desdimethyl B_{12} in certain microorganisms and in pernicious anemia patients (preliminary report(1,2)), it is obvious that the compound has only a small amount of activity for the chick.

Since this work was completed, we have

* Diet $C17B_{12}$ is the same as diet C8(6) minus vit. B_{12} with 20% of lard substituted for an equal amount of corn meal. It is composed of the following per kg: soybean oil meal 350 g, ground yellow corn 375 g, lard 200 g, glucose 10 g (containing riboflavin 8 mg), corn oil 5 g (containing vit. D_3 0.02 mg), and chick salts A 60 g.

† Purchased from Merck and Co., Rahway, N. J.

‡ Glaxo Laboratories, Ltd., Sefton Park, Stoke Poges, Bucks, England.

TABLE I. Growth of Chicks Fed Graded Levels of Desdimethyl B_{12} and Vit. B_{12}.*

γ of supplement/kg of basal diet		Avg wt in g at 4 wk and S.E.† Series 91 ♀	Series 92 ♀	Series 93 ♂	Series 94 ♀	Avg % increase in wt‡	Activity of desdimethyl B_{12} (%)§
	0	154 ± 18	145 ± 23	118 ± 15	158 ± 24	0	—
Vit. B_{12}	5	178 ± 27	—	221 ± 12	—	39	—
	10	286 ± 9	279 ± 20	248 ± 20	266 ± 17	85	—
	20	—	—	—	277 ± 27	80	—
	50	244 ± 33	—	—	320 ± 19	94	—
	100	269 ± 14	311 ± 9	298 ± 25	306 ± 20	100	—
Desdimethyl B_{12}	5	162 ± 22	—	115 ± 22	—	3	10
	10	162 ± 14	146 ± 27	99 ± 5	174 ± 29	2	5
	10 inj.‖	—	170 ± 16	140 ± 27	—	14	(20)
	20	—	—	—	181 ± 39	16	8
	50	—	—	—	268 ± 30	74	16
	100	265 ± 20	—	—	232 ± 17	74	6
							Avg = 9

* 6 chicks started/group. Mortality negligible.

† S.E. determined by method of Mantel(8).

‡ Increased in weight over basal group (0 γ B_{12}) with 100 γ B_{12} group = 100%. Calculations made for each series and then averaged.

§ An approximate calculation extrapolated from a curve based on the values in the preceding column. Final values corrected for presence of 3% of vit. B_{12} as an impurity in desdimethyl B_{12}.

‖ Injections made 3 times/wk. Amount of desdimethyl B_{12} administered was equal to amount of desdimethyl B_{12} consumed during preceding 2 or 3 day period by the group receiving 10 γ desdimethyl B_{12} in the diet.

learned that similar growth results with this compound have been obtained in independent studies with the chick by Dr. M. E. Coates of Shinfield, England(3). She has found that the compound had about 27% of the activity of vit. B_{12}.

Summary. Desdimethyl B_{12} had only approximately 9% of the activity of vit. B_{12} in stimulating growth of chicks fed a vit. B_{12}-low, corn-soybean diet containing 20% of lard.

We wish to thank Mrs. Ligia O. Ortiz and Mr. C. E. Emery for helpful technical assistance in these studies.

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Method for Measuring Mechanical Fragility of Dog Red Cells.* (21798)

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Increased susceptibility of red blood cells to lysis by mechanical trauma is of physiologic significance in certain hemolytic anemias(1,2). Shen *et al.*(1) quantitated the mechanical fragility (MF) of red blood cells by measuring the percentage release of hemoglobin that results when whole blood is rotated with glass beads under standard conditions. When this method is applied to canine blood, the MF of the erythrocytes of normal dogs varies greatly (Table I) and may not correlate with other signs of increased rate of red blood cell destruction *in vivo*(3,4). Stewart and his associates(5) also noted this phenomenon and found that the MF of dog red cells suspended in saline was more reproducible than if red cells were suspended in serum or plasma. The following observations were made on dog blood in the course of studies of the hemolytic effects of phenylhydrazine and led to the development of a satisfactory method for quantitating the MF of dog red cells.

Materials and methods. Five normal male mongrel dogs were used as a source of blood. The dogs were always fasted for at least 12 hours prior to being bled. The mechanical fragilities of red blood cells suspended in serum and saline were measured by a modification of the method of Shen *et al.*(1). For serum studies blood defibrinated by shaking with glass beads was adjusted to a hematocrit of 35% by adding or removing autologous serum. For saline studies blood was mixed with Alsever's Solution(6) in equal volumes immediately after being shed. The red cells were then washed once in isotonic saline and resuspended in isotonic saline so that the final hematocrit was 35%. Immediately after final mixing to secure uniformity, 0.5 ml of the adjusted red cell suspension either in serum or in saline was placed in a 50 ml Ehrlenmeyer flask with 10 glass beads uniformly 4 mm in diameter. The flask was sealed with a rubber stopper and attached by clamps to the outer rim of a wheel approximately 150 cm in diameter. The wheel was rotated at 30 rpm in an incubator at 37°C for 90 minutes in such a way that the beads rolled in the blood in the greatest internal circumference of the flask. Following this, the percentage of free hemoglobin in the rotated sample of red cell suspension (A) and in a control sample of red cell suspension which was placed in the same incubator but not rotated (B) were determined. This was done by adding 0.1 ml of each red cell suspension to separate 1 ml volumes of 1.25% sodium chloride solution. After brief centrifugation, hemoglobin concentration in supernatant fluid was measured with an Evelyn colorimeter. The value for complete hemolysis(C) was found by measuring the hemoglobin concentration in supernatant of a mixture of 0.1 ml of red cell suspension and 1 ml of distilled water. The percentage of the total hemoglobin released from red cells by trauma or the MF was calculated from the formula: $\text{MF} = \frac{\text{A-B}}{\text{C-B}} \times 100$. In experiments where blood was incubated prior to determination of its MF this was done under sterile conditions at room temperature. Hemoglobin levels, hematocrits and reticulocyte counts were measured by standard methods(7). Wet preparations of blood were stained by the method of Webster *et al.*(8) to show Heinz bodies. The red cells containing Heinz bodies per thousand red cells were counted and the percentage of cells containing Heinz bodies computed. Methemoglobin was measured by the method of Evelyn and Malloy(9) and expressed as percentage of total hemoglobin.

* This investigation was supported in part by a research grant RG-3507 from the National Institutes of Health, Public Health Service.

† Public Health Service Research Fellow of the National Heart Institute.

TABLE I. Comparison of Mechanical Fragility of Dog Red Cells Suspended in Autologous Serum with That of an Aliquot of the Same Erythrocytes Suspended in Isotonic Saline.

Dog	MF of erythrocytes, % In serum	In saline	Incubation,* min.
M-1	31.5	4.5	105
1	32.5	1.5	60
1	22.8	2.4	?
1	30.0	3.0	60
1	9.9	4.8	65
1	5.6	1.7	60
1	27.0	3.4	60
2	44.5	8.0	60
2	52.9	5.3	60
3	10.7	3.0	65
4	16.0	3.5	?
5	7.3	7.9	?
Mean	24.2%	4.1%	
Stand. dev.	± 15.0%	± 2.1%	

* Time elapsing between shedding of blood and exposure to mechanical trauma. During this time the blood was at room temperature.

Results. 1. The MF of dog red cells from freshly drawn blood suspended in autologous serum was compared with that of simultaneously drawn red cells suspended in isotonic saline. 12 such observations were made on blood from 5 dogs and the results tabulated in Table I. The mean MF of dog red cells suspended in serum was 24.2%. The range was 5.6% to 52.9% and the standard deviation was 15.0%. The mean MF of dog red cells suspended in saline was 4.1%. The range was 1.5% to 8.0% and the standard deviation was 2.1%.

2. Incubation of dog red cells suspended in autologous serum at 20°C resulted in a decrease of their MF during the first few hours. Generally, after about 3 to 7 hours at room temperature, the MF of the sample was less than 5% and approximated that of washed dog red cells suspended in saline. The results of a typical incubation experiment are plotted in Fig. 1.

3. Heparinized plasma and oxalated plasma had the same effect upon MF of the dog red cells as serum. Dog red cells suspended in fresh normal human serum had the same MF as when suspended in fresh autologous serum. Normal human erythrocytes suspended in fresh normal canine serum had the low MF characteristic of human red cells suspended in autologous serum.

4. Heating dog serum to 60°C for 30 minutes did not remove the factor which causes a high initial MF of dog red cells. This factor disappeared from serum which was separated from red cells and incubated at 20°C for 5 hours.

5. A normal dog was given subcutaneous injections of 20 mg of phenylhydrazine/kg body weight on each of 2 successive days. The MF of this dog's red cells measured simultaneously in serum and in saline, the circulating hemoglobin level, the percentages of red cells containing Heinz bodies, of total pigment present as methemoglobin and of reticulocytes are charted in Table II. The MF of this dog's red cells suspended in serum varies greatly from day to day and does not correlate with the degree of anemia or other red cell alterations. On the other hand, the MF of its red cells suspended in saline is more uniform and is highest shortly after administration of the hemolytic agent at a time when there is other evidence of red cell injury; *e.g.*, falling circulating hemoglobin level and the presence of Heinz bodies and of methemoglobin.

Discussion. Normal fresh fasting dog plasma or serum contains a factor which causes high initial values for the MF of autologous red cells when these are suspended therein. This factor is also present in fresh normal human serum but does not affect the MF of human erythrocytes. Incubation of

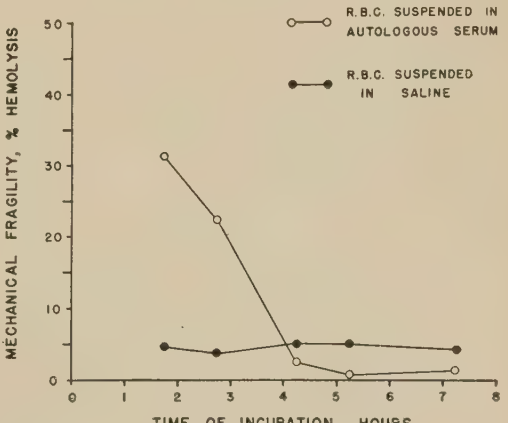

FIG. 1. Effect of incubation at 20°C upon the MF of dog red cells suspended in autologous serum and that of an aliquot of the same red cells suspended in isotonic saline.

TABLE II. Effect of the Administration of Phenylhydrazine* to a Normal Dog.

Day	Hemoglobin, g %	Reticulocytes, %	RBC containing Heinz bodies, %	Methemoglobin, %	MF of erythrocytes, %†	
					In serum	In saline
0	13.6	1.2	0	0.7	52.9	5.3
1	10.9	1.9	41	2.4	21.2	11.5
2	9.4	4.7	100	13.2	51.4	11.7
3	7.6	5.4	100	5.2	25.1	11.0
5	5.1	13.9	59	2.6	2.0	8.8
6	4.4	13.7	19	2.3	24.2	8.4
7	4.3	12.3	20		5.7	4.4
9	4.8	19.6	2		29.1	5.2
12	6.2	16.4	0		61.8	4.3
14	7.0	11.0			13.4	4.7
17	7.5	14.0			67.1	6.7
22	8.9	4.0			33.1	2.9
28	10.0	2.1			49.7	2.3
35	10.5	2.9			67.6	3.6
42	12.1	2.6			61.4	3.1
49	13.5	.5			19.2	6.2
63	13.2	.1			61.2	1.2

* Dog was given 20 mg of phenylhydrazine/kg of body wt subcut. on day 0 and on day 1.
† Measured 1-2 hr after blood was drawn from dog.

dog red cells in autologous serum or of dog serum alone at room temperature causes this factor to disappear after a period of several hours. The low MF of such red cells after 3 to 7 hours' incubation is not due to hemolysis of the most susceptible cells since gross hemolysis is not noted in the incubated blood. Swank and Roth(10) noted increased MF of dog red cells suspended in plasma and believed that this correlated with the degree of lipemia that appeared following fatty meals. In our experiments the dogs were always in a fasting state when blood was drawn and lipemia was not observed. The factor causing the high initial MF has not been identified or isolated. It apparently has no significant effect upon the survival of dog red cells *in vivo* and none of the dogs studied was anemic. These phenomena concerning dog red cells have not been noted in human red cells and thus indicate a difference in the behavior of the erythrocytes of these species under the conditions defined above.

Summary. Large variations in the MF of freshly drawn samples of defibrinated, oxalated or heparinized dog blood are encountered unless the samples are allowed to stand for several hours at room temperature. However, reproducible and apparently physiologically significant measurements of the MF of dog red cells can be obtained if the red cells are washed free of plasma and suspended in isotonic saline during their exposure to the standard trauma of rotation with glass beads.

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Antibody Production to Diphtheria Toxoid in Vitamin Deficiency States.* (21799)

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The deleterious effect of certain specific vitamin deficiencies in the rat on the production of antibodies to human erythrocytes has been demonstrated(1). Since antigens vary in their immunological behavior, it seemed appropriate to extend these observations to the effects of other antigenic stimuli in some of the same vitamin deficiency states. Purified diphtheria toxoid was chosen for this purpose. The utilization of this antigen would also relate our studies more closely to the effects of vitamin deficiencies on resistance to infection. The present paper reports the effects of pantothenic acid, riboflavin, pyridoxine, biotin, thiamine, pteroylglutamic acid (PGA), vit. A, and vit. D deficiencies on the ability of the rat to produce circulating antibodies to purified diphtheria toxoid.

Methods. Animals and diets. Male, weanling, albino rats of the Sprague-Dawley strain were employed. The animals were housed individually in wide-meshed screen bottom cages. The composition of the basal diet fed each group is shown in Table I. A control group for each of the deficiencies received the same basal diet as the corresponding deficient group. All animals were fed *ad libitum* with the exception of a group of inanition controls which was paired-fed with the riboflavin-deficient animals. In addition, each animal received a vitamin pill daily. Each of the pills fed the control groups and the vit. A- and vit. D-deficient groups supplied the following vitamins: thiamine, 40 γ; niacin, 100 γ; riboflavin, 60 γ; calcium pantothenate, 300 γ; pyridoxine, 50 γ; biotin, 4 γ; PGA, 2 γ. For each of the deficient groups the appropriate vitamin was omitted from the pill. Because of the high mortality rate in the thiamine-deficient animals, it was found necessary to supplement this group with 40 γ of thiamine daily for the first 6 days of the experiment. These animals gained weight steadily until they reached approximately 100 g 4 days prior to immunization. The weights then leveled off and declined steadily for the remainder of the

TABLE I. Composition of the Basal Diets.

Component	Regular*	PGA	Biotin	Vit. A†	Vit. D‡
Casein "vit. free"	25.0	25.0	10.0	25.0	—
Dried egg white	—	—	15.0	—	—
Sucrose	56.8	56.3	56.8	60.8	—
Hydrogenated vegetable oil	10.0	10.0	10.0	—	—
Cod liver oil	2.0	2.0	2.0	—	—
Corn oil	2.0	2.0	2.0	—	—
Cottonseed oil	—	—	—	10.0	—
Salts(2)	4.0	4.0	4.0	4.0	—
Sulfasuxidine	—	.50	—	—	—
Choline chloride	.20	.20	.20	.20	—
i-inositol	.03	.03	.03	.03	—
dl-α-tocopherol acetate	.01	.01	.01	.01	—
Menadione	.001	.001	.001	.001	—
Whole ground yellow corn	—	—	—	—	76.
Ground gluten	—	—	—	—	20.
Calcium carbonate	—	—	—	—	3.
Sodium chloride	—	—	—	—	1.

* Basal diet for pantothenic acid, pyridoxine, riboflavin and thiamine groups.

† Control and deficient animals received 250 U.S.P. units of calciferol weekly *per os*. Controls also received 6000 U.S.P. units of vit. A acetate weekly *per os*.

‡ Controls received *per os* 250 units of calciferol weekly.

* This study was supported by a grant from the National Vitamin Foundation, N. Y. City.

TABLE II. Body Weights of Vitamin Deficient Rats and Their Controls.*

Group	Deficient		Control	
	Initial	Final†	Initial	Final†
	g			
Pantothenic acid	48	110	48	308
Pyridoxine	46	170	45	345
Riboflavin	45	54	45	345
Biotin	47	159	47	340
PGA	45	110	45	187
Thiamine	46	68	46	166
Vit. A	45	87	45	166
" D	46	66	46	63

* Group averages.
† At the time of the serum antibody titrations.

experiment. *Immunization.* The pantothenic acid, pyridoxine, riboflavin and biotin groups were immunized after 4 weeks on the appropriate diets; the vit. D, PGA and thiamin groups after 2 weeks; and the vit. A group after 12 days. All animals received one intraperitoneal injection of 0.15 ml of alum-precipitated diphtheria toxoid (Lederle). Two and one-half to 3 weeks later they were placed under nembutal anesthesia and bled by cardiac puncture. Sera were heated at 56°C for ½ hour, absorbed at room temperature with an equal volume of sheep erythrocytes and stored in the deep freeze until titration. The anamnestic response of the PGA group to diphtheria toxoid was determined by injecting the animals intraperitoneally with 0.15 ml of the antigen on the day after the initial bleeding and determining the serum antibody titer one week later. *Antibody titration.* Antibody titers were determined by hemagglutination of sheep red cells treated with tannic acid and coated with fluid diphtheria toxoid (Lederle).† The procedure was that of Stavitsky(3) who applied the method of Boyden(4) to diphtheria toxoid.

Results. The growth records of the animals are summarized in Table II. Individual antibody titers to purified diphtheria toxoid in the deficiencies studied are shown in Fig. 1, 2, and 3. Impairment of antibody response was noted in pantothenic acid, riboflavin, pyridoxine, biotin and vit. D deficiencies. A less pronounced effect was observed in vit. A deficiency. No impairment of antibody response was evidenced in the thiamine-deficient group.

† Generously supplied by Lederle Laboratories.

The results obtained in the PGA group require special comment. Neither a primary nor a secondary response to the antigen was elicited in the deficient group. A large proportion of the control animals also failed to produce antibody to the primary antigenic stimulus despite their adequate intake of pteroylglutamic acid. It seems that the sulfasuxidine in the diet exerted a deleterious effect upon the antibody producing systems in addition to its bacteriostatic effect on the intestinal microorganisms involved in PGA synthesis. The mechanism of this action of sulfasuxidine is not known. No other symptoms of drug toxicity were apparent. In contrast to the deficient group a number of PGA control animals responded anamnestically to diphtheria toxoid. Some of these animals did not show a primary response.

The normal antibody production of the inanition controls was a further demonstration of the failure of inanition *per se* to affect the immune response.

Discussion. In preliminary experiments it was noted that many vit. A control rats were unable to produce antibody. With continued

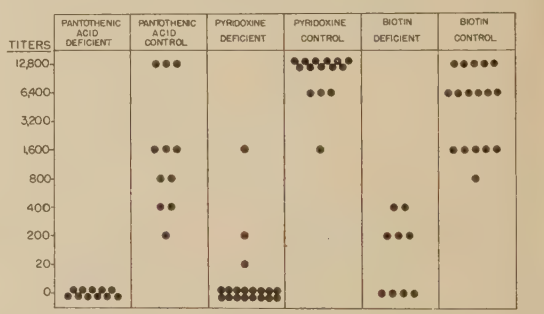

FIG. 1. Individual antibody titers to diphtheria toxoid.

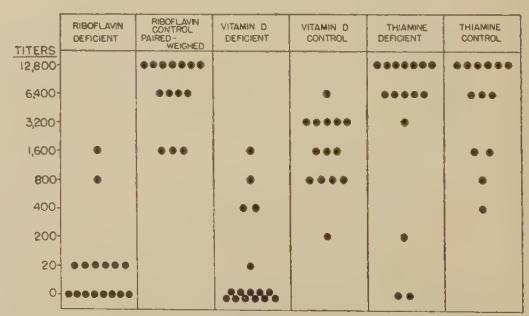

FIG. 2. Individual antibody titers to diphtheria toxoid.

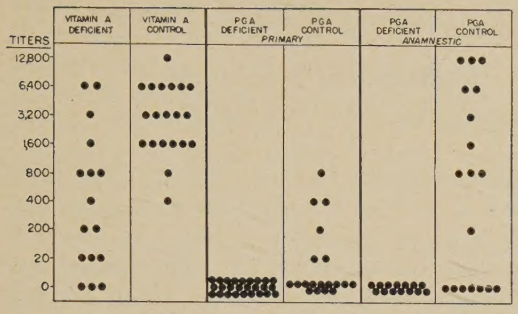

FIG. 3. Individual antibody titers to diphtheria toxoid.

experimentation it was found that the longer these animals were maintained on the basal diets prior to immunization, the more pronounced was their inability to synthesize antibody. These controls grew normally and no other deficiency signs were manifested. It will be noted that the vit. A group described in the text was on experiment only 12 days prior to immunization. As seen in Fig. 3, these control rats showed a normal antibody response. It is possible that a factor necessary for antibody production but not for growth is absent from the basal diet and may become limiting for antibody production as the animals are maintained for longer periods on this regimen. This would represent another example of the lack of correlation between a dietary requirement for growth and that for antibody production(5) and would illustrate the value of utilizing antibody production as a criterion of nutritional adequacy.

In general, the effects of the vitamin deficiency states reported here were similar to those previously obtained using human erythrocytes as antigen. In the present study using purified diphtheria toxoid as antigen a more pronounced impairment of antibody response was observed in riboflavin and biotin deficiencies. Most noteworthy was the deleterious effect of a vit. D deficiency with this antigen. In previous experiments using human red cells(6) no adverse effect of this deficiency was noted. To the authors' knowledge there is no other reported instance of a harmful effect of vit. D deficiency upon antibody production. This variance in results with the two antigens is an interesting phenomenon and invites further investigation.

The significance of certain vitamins in antibody production is further illustrated in these experiments with diphtheria toxoid. The use of this antigen has emphasized the possible role of vitamins in establishing a satisfactory degree of actively acquired immunity. A comprehensive summary of the experimental work dealing with the relationship of vitamins to antibody response has been presented elsewhere(5).

Summary. Antibody response to purified diphtheria toxoid in vitamin deficient rats and their respective controls has been determined. Impairment of antibody response was noted in pantothenic acid, pyridoxine, pteroylglutamic acid, biotin, riboflavin and vit. D deficiencies. Vit. A deficiency had only a moderate effect and thiamine-deficient rats showed normal antibody titers.

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Proliferation of Monkey Kidney Cells in Rotating Cultures. (21800)

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Our object in beginning this work was to devise a technic for the propagation of animal cells in tissue culture similar to that used for bacterial cultures. Such a technic would permit the precise experimental approach possible with many bacterial species to be applied to a number of biochemical studies on animal cells. It has already been shown that several strains of animal cells will multiply while suspended in a nutrient medium if the culture is agitated continuously(1-4). To use this procedure for certain biochemical experiments it seemed desirable to eliminate, if possible, the initial lag in multiplication(1,2) and to obtain a greater number of cells during the logarithmic phase of multiplication (1,2,4). By using a strain of monkey kidney cells we have found it possible to achieve a 100-fold increase in cell population during the logarithmic phase of multiplication without an early lag.

Methods. The strain of cells was supplied by Dr. Raymond C. Parker of this institution. Originally isolated from monkey kidney cortex, the cells had undergone a transformation during maintenance in his laboratory(5). The altered cells were small (12-15 μ), spherical in shape, and showed a tendency to float free of the glass and multiply in the nutrient medium even in stationary flasks. The medium in our experiments consisted of 2% chick embryo extract and 40% horse serum in Earle's balanced saline solution, and contained per ml 200 μg dihydrostreptomycin sulfate and 500 units potassium penicillin G. Cultures were incubated at 37°C in glass or silicone(6) stoppered test tubes of various sizes rotated horizontally around their long axes at 40-50 rpm. The cells showed no tendency to stick to the glass. At intervals, small aliquots were withdrawn, a drop of 1% methylene blue was added and the cells enumerated in a standard bacterial counting chamber or hemocytometer.

Results. When the cells were subcultured from stationary flasks to rotating tubes there was a decrease in population during the first 24 hours as observed by Earle *et al.* for the L strain of mouse cells(1,2). This interval was followed by a period during which the cell number increased logarithmically. The initial delay in cell multiplication was not affected by addition to the culture of various proportions of "conditioned" medium, *i.e.*, medium that had already supported the growth of several generations of cells. It was eliminated, however, if cells already in the logarithmic phase in rotating cultures were used for the subculture.

The following experiment illustrates some of the properties of this strain. Cells from a rotated culture in the logarithmic phase were inoculated into fresh medium at a concentration of 2.3 x 10^5 cells per ml, the final volume being about 15 ml in a 25 ml tube. This culture was rotated and aliquots were removed at intervals for cell counts. The results are shown in Fig. 1. When the cell concentration reached about 2 x 10^6 per ml, the culture was divided into 2 parts. The first part was centrifuged lightly, about two-thirds of the supernatant was withdrawn, replaced with fresh

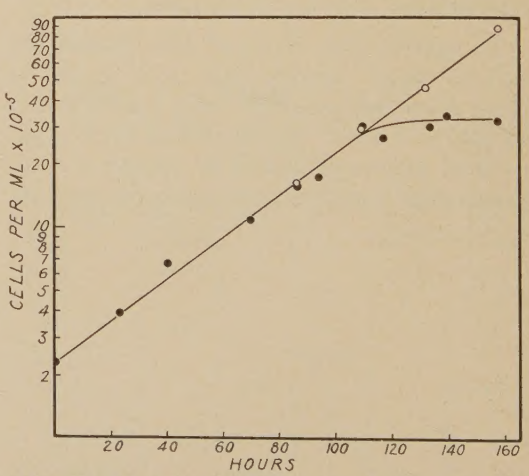

FIG. 1. Multiplication of monkey kidney cells in rotating culture at 37°C.

medium and the culture again rotated. At succeeding 24 hourly intervals the medium was renewed as before. Cell increase in this culture is represented by the open circles in Fig. 1. The second part of the culture was allowed to continue without further treatment; its behaviour is represented by the closed circles in Fig. 1.

It is observed that the increase in cell number was logarithmic from time zero with a generation time of 31 hours. Multiplication continued to a density 3 x 10^6 per ml and then stopped. When part of the medium was replaced at intervals, multiplication continued to about 10^7 cells per ml without departing from linearity. Other experiments have shown that the same type of growth curve may be obtained with initial cell densities as low as 10^5 per ml; when the initial concentration was 4 x 10^4 cells per ml, a lag period was observed before multiplication began.

The foregoing experiments show that at least a 100-fold increase in cells during the logarithmic phase may be obtained. The generation time under these conditions is 30-36 hours and is reproducible from one experiment to another. In contrast, the generation time in stationary cultures was 70-90 hours. It should be noted that the generation time is a sensitive indicator of the physiological state of the cells and is a logical value for use in assessing the action of nutritional supplements, metabolic inhibitors or, in some cases, virus infection, on the cells.

Stock cultures of these cells were kept continuously in the logarithmic phase for over 2 months. Aliquots were removed from time to time and replaced with fresh medium. The concentrations in stock cultures were not allowed to exceed 2.5 x 10^6 or fall below 10^5 cells per ml, but no regular schedule for changing medium was observed. There seems to be no obvious restriction on the size of cultures that may be grown, but to ensure adequate oxygen supply the culture volume should probably not occupy more than two-thirds the volume of the container. In fact, this strain of cells would seem to be eminently suitable for use in a continuous culture type of apparatus based on the principle of the chemostat(7,8).

Summary. A method is described for the propagation of a strain of cells from monkey kidney in tubes rotated around their horizontal axes at 40-50 rpm. Under these conditions, it was possible to achieve a 100-fold increase in the logarithmic phase without initial lag. The generation time was about 33 hours.

We wish to acknowledge our gratitude to Dr. Parker and his associates for much discussion and advice during this work. The research was aided by grants from the National Cancer Institute of Canada, the National Institutes of Health of the United States (Public Health Service), and the W. B. Boyd Memorial Fund.

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